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(71) Applicant *(for all designated States except US)*: **CARGILL, INCORPORATED** [US/US]; P.O. Box 5624, Minneapolis, MN 55440-5624 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **DE SOUZA, Mervyn, L.** [US/US]; Apartment 10, 1029 Raymond Avenue, St. Paul, MN 55114-1134 (US). **KOLLMANN, Sherry, R.** [US/US]; 12031 99th Avenue North, Maple Grove, MN 55369 (US). **MAY, Colleen, A.** [US/US]; 20 Gideons Point Road, Tonka Bay, MN 55331 (US). **SCHROEDER, William, A.** [US/US]; 3509 Highlands Road, Brooklyn Park, MN 55443 (US).

(74) Agent: **DEGRANDIS, Paula**; Cargill, Incorporated, P.O. Box 5624, Minneapolis, MN 55440-5624 (US).

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(54) Title: CAROTENOID BIOSYNTHESIS

(57) Abstract: Membranous bacteria that produce astaxanthin and other carotenoids are described, as well as isolated nucleic acids and expression vectors that can be used for producing carotenoids in microorganisms.

Carotenoid Biosynthesis

TECHNICAL FIELD

The invention relates to methods and materials for producing carotenoids, and in particular, to nucleic acid molecules, polypeptides, host cells, and methods that can be used for producing carotenoids.

BACKGROUND

Astaxanthin (3,3'-dihydroxy-β,β-carotene-4,4'-dione) is the primary carotenoid that imparts the pink pigment to the eggs, flesh, and skin of salmon, trout, and shrimp. Most animals cannot synthesize carotenoids. Rather, the pigments are acquired through the food chain from marine algae and phytoplankton, the primary producers of astaxanthin. ATX exists in three configurational isomers [(3S, 3'S), (3R, 3'R) and (3S, 3'R; 3R, 3'S)], however, ATX is found in the marine environment only in the (3S, 3'S) form. Consequently, this form is considered the natural and most desirable form of ATX.

Although astaxanthin has been commercially extracted from some yeast and crustacea species and has been chemically synthesized as a 1:2:1 mixture of the (3S,3'S)-, (3S,3'R)- and (3R,3'R)-isomers, astaxanthin is limited in availability and is expensive to purchase. See, Torrisen et al. (1989) Crit. Rev. Aquatic Sci. 1:209; and Mayer (1994) Pure Appl. Chem., 66:931-938. Thus, there is a need for a less expensive source of the naturally-occurring (3S,3'S) astaxanthin.

SUMMARY

The invention is based on methods and materials for producing carotenoids such as lycopene, zeaxanthin, zeaxanthin diglucoside, canthaxanthin, β-carotene, lutein, and astaxanthin. Such carotenoids can be used as nutritional supplements in humans and can be formulated for use in aquaculture or as an animal feed. The invention provides nucleic acid molecules that can be used to engineer host cells having the ability to produce particular carotenoids and polypeptides that can be used in cell-free systems to make particular carotenoids. The engineered cells described herein can be used to produce large quantities of carotenoids.

In one aspect, the invention features an isolated nucleic acid having at least 76% sequence identity to the nucleotide sequence of SEQ ID NO:1 (e.g., at least 80%, 85%, 90%, or 95% sequence identity to the nucleotide sequence of SEQ ID NO:1) or to a fragment of SEQ ID NO:1 at least 33 contiguous nucleotides in length. An isolated nucleic acid can encode a zeaxanthin glucosyl transferase polypeptide at least 75% identical to the amino acid sequence of SEQ ID NO:2. Expression vectors containing such nucleic acids operably linked to an expression control element also are featured.

In another aspect, the invention features an isolated nucleic acid having at least 78% sequence identity to the nucleotide sequence of SEQ ID NO:3 (e.g., at least 80%, 85%, 90%, or 95% sequence identity to the nucleotide sequence of SEQ ID NO:3) or to a fragment of SEQ ID NO:3 at least 32 contiguous nucleotides in length. An isolated nucleic acid can encode a lycopene β-cyclase polypeptide at least 83% identical to the amino acid sequence of SEQ ID NO:4. β-carotene can be made by contacting lycopene with a polypeptide encoded by such isolated nucleic acids. The invention also features an expression vector that includes such nucleic acids operably linked to an expression control element.

In yet another aspect, the invention features an isolated nucleic acid having at least 81% sequence identity to the nucleotide sequence of SEQ ID NO:5 (e.g., at least 85%, 90%, or 95% sequence identity to the nucleotide sequence of SEQ ID NO:5) or to a fragment of SEQ ID NO:5 at least 60 contiguous nucleotides in length. An isolated nucleic acid also can encode a geranylgeranyl pyrophosphate synthase polypeptide at least 85% identical to the amino acid sequence of SEQ ID NO:6. Geranylgeranyl pyrophosphate can be made by contacting farnesyl pyrophosphate and isopentenyl pyrophosphate with a polypeptide encoded by such nucleic acids. Expression vectors that include such nucleic acids operably linked to an expression control element also are featured.

Isolated nucleic acids having at least 82% sequence identity to the nucleotide sequence of SEQ ID NO:7 (e.g., at least 85%, 90%, or 95% sequence identity to the nucleotide sequence of SEQ ID NO:7) or to a fragment of SEQ ID NO:7 at least 30 contiguous nucleotides in length also are featured. An isolated nucleic acid also can encode a phytoene desaturase polypeptide at least 90% identical to the amino acid

sequence of SEQ ID NO:8. Lycopene can be made by contacting phytoene with a polypeptide encoded by such nucleic acids. An expression vector that includes such nucleic acids operably linked to an expression control element also is featured.

The invention also features an isolated nucleic acid having at least 82% sequence identity to the nucleotide sequence of SEQ ID NO:9 (e.g., at least 85%, 90%, or 95% sequence identity to the nucleotide sequence of SEQ ID NO:9) or to a fragment of SEQ ID NO:9 at least 23 contiguous nucleotides in length. An isolated nucleic acid also can encode a phytoene synthase polypeptide at least 89% identical to the amino acid sequence of SEQ ID NO:10. Phytoene can be made by contacting geranylgeranyl pyrophosphate with a polypeptide encoded by such nucleic acids. An expression vector that includes such nucleic acids operably linked to an expression control element also is featured.

In yet another aspect, the invention features an isolated nucleic acid having at least 85% sequence identity to the nucleotide sequence of SEQ ID NO:11 (e.g., at least 90% or 95% identity to the nucleotide sequence of SEQ ID NO:11) or to a fragment of SEQ ID NO:11 at least 36 contiguous nucleotides in length. An isolated nucleic acid can encode a β-carotene hydroxylase polypeptide at least 90% identical to the amino acid sequence of SEQ ID NO:12. Zeaxanthin can be made by contacting β-carotene with a polypeptide encoded by such nucleic acids. Astaxanthin can be made by contacting canthaxanthin with a polypeptide encoded by such nucleic acids. The invention also features an expression vector that includes such nucleic acids operably linked to an expression control element.

The invention also features membranous bacteria (e.g., a *Rhodobacter* species) that include at least one exogenous nucleic acid encoding phytoene desaturase, lycopene β-cyclase, β-carotene hydroxylase, and β-carotene C4 oxygenase, wherein expression of the at least one exogenous nucleic acid produces detectable amounts of astaxanthin in the membranous bacteria. The amino acid sequence of the phytoene desaturase can be at least 90% identical to the amino acid sequence of SEQ ID NO:8. The amino acid sequence of the lycopene β-cyclase can be at least 83% identical to the amino acid sequence of SEQ ID NO:4. The amino acid sequence of the β-carotene hydroxylase can be at least 90% identical to the amino acid sequence of SEQ ID NO:12. The amino acid sequence of the β-carotene C4 oxygenase can be at least 80% identical to the amino acid

sequence of SEQ ID NO:39. The membranous bacteria further can include an exogenous nucleic acid encoding geranylgeranyl pyrophosphate synthase (e.g., a multifunctional geranylgeranyl pyrophosphate synthase) or can lack endogenous bacteriochlorophyll biosynthesis. The multifunctional geranylgeranyl pyrophosphate synthase can have an amino acid sequence at least 90% identical to the amino acid sequence of SEQ ID NO:45. The membranous bacteria further can include an exogenous nucleic acid encoding phytoene synthase. The phytoene synthase can have an amino acid sequence at least 89% identical to the amino acid sequence of SEQ ID NO:10.

In another aspect, the invention features membranous bacteria that include an exogenous nucleic acid encoding a phytoene desaturase having an amino acid sequence at least 90% identical to the amino acid sequence of SEQ ID NO:8, and wherein the membranous bacteria produces detectable amounts of lycopene. The membranous bacteria further can include a lycopene β-cyclase, wherein the membranous bacteria produce detectable amounts of β-carotene. The membranous bacteria also can include a β-carotene hydroxylase, wherein the membranous bacteria produce detectable amounts of zeaxanthin.

In still yet another aspect, the invention feature membranous bacteria that include at least one exogenous nucleic acid encoding phytoene desaturase, lycopene β-cyclase, and β-carotene C4 oxygenase, wherein expression of the at least one exogenous nucleic acid produces detectable amounts of canthaxanthin in the membranous bacteria. The membranous bacteria also can include a β-carotene hydroxylase, wherein the membranous bacteria produce detectable amounts of astaxanthin.

The invention also features a composition that includes an engineered *Rhodobacter* cell, wherein the cell produces a detectable amount of astaxanthin or canthaxanthin. The engineered *Rhodobacter* cell can include at least one exogenous nucleic acid encoding phytoene desaturase, lycopene β-cyclase, β-carotene hydroxylase, and β-carotene C4 oxygenase. The composition can be formulated for aquaculture and can pigment the flesh of fish or the carapace of crustaceans after ingestion. The composition can be formulated for human consumption or as an animal feed (e.g., formulated for consumption by chickens, turkeys, cattle, swine, or sheep).

The invention also features a method of making a nutraceutical. The method includes extracting carotenoids from an engineered *Rhodobacter* cell, the engineered *Rhodobacter* cell including at least one exogenous nucleic acid encoding phytoene desaturase, lycopene β-cyclase, β-carotene hydroxylase, and β-carotene C4 oxygenase, and wherein the *Rhodobacter* cell produces detectable amounts of astaxanthin.

In yet another aspect, the invention features membranous bacteria, wherein the membranous bacteria include an exogenous nucleic acid encoding a lycopene β-cyclase having an amino acid sequence at least 83% identical to the amino acid sequence of SEQ ID NO:4. The membranous bacteria further can include a phytoene desaturase, (e.g., an exogenous phytoene desaturase), wherein the membranous bacteria produce detectable amounts of β-carotene. The membranous bacteria also can include a β-carotene hydroxylase (e.g., an exogenous β-carotene hydroxylase), wherein the bacteria produce detectable amounts of zeaxanthin.

Membranous bacteria that include a β-carotene hydroxylase having an amino acid sequence at least 90% identical to the amino acid sequence of SEQ ID NO:12 also is featured. The membranous bacteria further can include a lycopene β-cyclase (e.g., an exogenous lycopene β-cyclase), wherein the membranous bacteria produce detectable amounts of zeaxanthin. The membranous bacteria also can include a phytoene desaturase (e.g., an exogenous phytoene desaturase), wherein the membranous bacteria produce detectable amounts of β-carotene.

The invention also features membranous bacteria (e.g., a *Rhodobacter* species) lacking an endogenous nucleic acid encoding a farnesyl pyrophosphate synthase, wherein the bacteria produces detectable amounts of carotenoids. The membranous bacteria also can include an exogenous nucleic acid encoding a multifunctional geranylgeranyl pyrophosphate synthase.

In another aspect, the invention features an isolated nucleic acid having at least 70% sequence identity (e.g., at least 80% or 90%) to the nucleotide sequences of SEQ ID NO:38, or to a fragment of the nucleic acid of SEQ ID NO:38 at least 15 contiguous nucleotides in length. The nucleic acid can encode a β-carotene C4 oxygenase. Canthaxanthin can be made by contacting β-carotene with a polypeptide encoded by such nucleic acids or a polypeptide having an amino acid sequence at least 80% identical to the

amino acid sequence of SEQ ID NO:39. Astaxanthin can be made by contacting zeaxanthin with a polypeptide encoded by such isolated nucleic acids or a polypeptide having an amino acid sequence at least 80% identical to the amino acid sequence of SEQ ID NO:39.

In another aspect, the invention features membranous bacteria that include an exogenous nucleic acid encoding a β-carotene C4 oxygenase, where the β-carotene oxygenase has an amino acid sequence at least 80% identical to the amino acid sequence of SEQ ID NO:39.

In yet another aspect, the invention features a host cell comprising an exogenous nucleic acid, wherein the exogenous nucleic acid includes a nucleic acid sequence encoding one or more polypeptides that catalyze the formation of (3S, 3'S) astaxanthin, wherein the host cell produces CoQ-10 and (3S, 3'S) astaxanthin. A method of making CoQ-10 and (3S, 3'S) astaxanthin at substantially the same time also is featured. The method includes transforming a host cell with a nucleic acid, wherein the nucleic acid includes a nucleic acid sequence that encodes one or more polypeptides, wherein the polypeptides catalyze the formation of (3S, 3'S) astaxanthin; and culturing the host cell under conditions that allow for the production of (3S, 3'S) astaxanthin and CoQ-10. The method further can include transforming the host cell with at least one exogenous nucleic acid, the exogenous nucleic acid encoding one or more polypeptides, wherein the polypeptides catalyze the formation of CoQ-10.

The invention also features isolated nucleic acid having a nucleotide sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:38, and SEQ ID NO:44.

An isolated nucleic acid having at least 90% sequence identity to the nucleotide sequences of SEQ ID NO:44, or to a fragment of the nucleic acid of SEQ ID NO:44 at least 60 contiguous nucleotides in length is featured. Geranylgeranyl pyrophosphate can be made by contacting isopentenyl pyrophosphate and dimethylallyl pyrophosphate with a polypeptide encoded by such a nucleic acid.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those

described herein can be used to practice the invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

DESCRIPTION OF DRAWINGS

FIG 1 is a schematic diagram of the biosynthetic pathway for the production of zeaxanthin and conversion to zeaxanthin di-glucoside.

FIG 2 is a schematic diagram of the *P. stewartii* carotenoid gene operon (6586 bp).

FIG 3 is a chromatogram of astaxanthin production in *P. stewartii::crtW*(*B. aurantiaca*).

DETAILED DESCRIPTION

Nucleic Acid Molecules

The invention features isolated nucleic acids that encode enzymes involved in carotenoid biosynthesis. The nucleic acids of SEQ ID NO:1, 3, 5, 7, 9, and 11 encode zeaxanthin glucosyl transferase (*crtX*), lycopene β-cyclase (*crtY*), geranylgeranyl-pyrophosphate synthase (*crtE*), phytoene desaturase (*crtI*), phytoene synthase (*crtB*) and β-carotene hydroxylase (*crtZ*), respectively. A nucleic acid of the invention can have at least 76% sequence identity, e.g., 78%, 80%, 85%, 90%, 95%, or 99% sequence identity, to the nucleic acid of SEQ ID NO:1, or to fragments of the nucleic acid of SEQ ID NO:1 that are at least about 33 nucleotides in length; at least 78% sequence identity, e.g., 80%, 85%, 90%, 95%, or 99% sequence identity, to the nucleotide sequence of SEQ ID NO:3, or to fragments of the nucleic acid of SEQ ID NO:3 that are at least about 32 nucleotides in length; at least 81% sequence identity, e.g., 82%, 85%, 90%, 95%, or 99% sequence identity, to the nucleotide sequence of SEQ ID NO:5 , or to fragments of the nucleic acid of SEQ ID NO:5 that are at least about 60 nucleotides in length; at least 82% sequence identity, e.g., 83%, 85%, 90%, 95%, or 99% sequence identity, to the nucleotide

sequences of SEQ ID NO:7 or SEQ ID NO:9 , or to fragments of the nucleic acids of SEQ ID NO:7 or SEQ ID NO:9 that are at least about 30 or 23 nucleotides in length, respectively; at least 85% sequence identity, e.g., 86%, 90%, 92%, 95%, or 99% sequence identity, to the nucleotide sequence of SEQ ID NO:11 , or to fragments of the nucleic acid of SEQ ID NO:11 that are at least about 36 nucleotides in length. A nucleic acid of the invention can have at least 60% sequence identity, e.g., at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 99% sequence identity to the nucleotide sequence of SEQ ID NO:38 or to fragments of the nucleic acid of SEQ ID NO:38 that are at least about 15 nucleotides in length. Such a nucleic acid can encode a β-carotene C4 oxygenase (*crtW*). A nucleic acid of the invention also can have at least 90% identity to the nucleotide sequence set forth in SEQ ID NO:44 or to fragments of the nucleic acid of SEQ ID NO:44 that are at least about 60 nucleotides in length. Such a nucleic acid can encode a multifunctional geranylgeranyl pyrophosphate synthase.

Generally, percent sequence identity is calculated by determining the number of matched positions in aligned nucleic acid sequences, dividing the number of matched positions by the total number of aligned nucleotides, and multiplying by 100. A matched position refers to a position in which identical nucleotides occur at the same position in aligned nucleic acid sequences. Percent sequence identity can be determined for any nucleic acid or amino acid sequence as follows. First, a nucleic acid or amino acid sequence is compared to the identified nucleic acid or amino acid sequence using the BLAST 2 Sequences (Bl2seq) program from the stand-alone version of BLASTZ containing BLASTN version 2.0.14 and BLASTP version 2.0.14. This stand-alone version of BLASTZ can be obtained from the University of Wisconsin library as well as at www.fr.com or www.ncbi.nlm.nih.gov. Instructions explaining how to use the Bl2seq program can be found in the readme file accompanying BLASTZ.

Bl2seq performs a comparison between two sequences using either the BLASTN or BLASTP algorithm. BLASTN is used to compare nucleic acid sequences, while BLASTP is used to compare amino acid sequences. To compare two nucleic acid sequences, the options are set as follows: -i is set to a file containing the first nucleic acid sequence to be compared (e.g., C:\seq1.txt); -j is set to a file containing the second nucleic acid sequence to be compared (e.g., C:\seq2.txt); -p is set to blastn; -o is set to any

desired file name (e.g., C:\output.txt); -q is set to –1; -r is set to 2; and all other options are left at their default setting. For example, the following command can be used to generate an output file containing a comparison between two sequences: C:\Bl2seq –i c:\seq1.txt –j c:\seq2.txt –p blastn –o c:\output.txt –q –1 –r 2. To compare two amino acid sequences, the options of Bl2seq are set as follows: -i is set to a file containing the first amino acid sequence to be compared (e.g., C:\seq1.txt); -j is set to a file containing the second amino acid sequence to be compared (e.g., C:\seq2.txt); -p is set to blastp; -o is set to any desired file name (e.g., C:\output.txt); and all other options are left at their default setting. For example, the following command can be used to generate an output file containing a comparison between two amino acid sequences: C:\Bl2seq –i c:\seq1.txt –j c:\seq2.txt –p blastp –o c:\output.txt. If the target sequence shares homology with any portion of the identified sequence, then the designated output file will present those regions of homology as aligned sequences. If the target sequence does not share homology with any portion of the identified sequence, then the designated output file will not present aligned sequences.

Once aligned, a length is determined by counting the number of consecutive nucleotides or amino acid residues from the target sequence presented in alignment with sequence from the identified sequence starting with any matched position and ending with any other matched position. A matched position is any position where an identical nucleotide or amino acid residue is presented in both the target and identified sequence. Gaps presented in the target sequence are not counted since gaps are not nucleotides or amino acid residues. Likewise, gaps presented in the identified sequence are not counted since target sequence nucleotides or amino acid residues are counted, not nucleotides or amino acid residues from the identified sequence.

The percent identity over a particular length is determined by counting the number of matched positions over that length and dividing that number by the length followed by multiplying the resulting value by 100. For example, if (1) a 1000 nucleotide target sequence is compared to the sequence set forth in SEQ ID NO:1, (2) the Bl2seq program presents 200 nucleotides from the target sequence aligned with a region of the sequence set forth in SEQ ID NO: 1 where the first and last nucleotides of that 200 nucleotide region are matches, and (3) the number of matches over those 200 aligned nucleotides is

180, then the 1000 nucleotide target sequence contains a length of 200 and a percent identity over that length of 90 (i.e. 180 ÷ 200 * 100 = 90).

It will be appreciated that a single nucleic acid or amino acid target sequence that aligns with an identified sequence can have many different lengths with each length having its own percent identity. For example, a target sequence containing a 20 nucleotide region that aligns with an identified sequence as follows has many different lengths including those listed in Table 1.

```
                    1                   20
Target Sequence:    AGGTCGTGTACTGTCAGTCA (SEQ ID NO:46)
                    |  ||  ||  |  ||||  ||  |  |
Identified Sequence: ACGTGGTGAACTGCCAGTGA (SEQ ID NO:47)
```

TABLE 1

Starting Position	Ending Position	Length	Matched Positions	Percent Identity
1	20	20	15	75.0
1	18	18	14	77.8
1	15	15	11	73.3
6	20	15	12	80.0
6	17	12	10	83.3
6	15	10	8	80.0
8	20	13	10	76.9
8	16	9	7	77.8

It is noted that the percent identity value is rounded to the nearest tenth. For example, 78.11, 78.12, 78.13, and 78.14 is rounded down to 78.1, while 78.15, 78.16, 78.17, 78.18, and 78.19 is rounded up to 78.2. It is also noted that the length value will always be an integer.

Isolated nucleic acid molecules of the invention are at least about 20 nucleotides in length. For example, the nucleic acid molecule can be about 20-30, 22-32, 33-50, 34 to 45, 40-50, 60-80, 62 to 92, 50-100, or greater than 150 nucleotides in length, e.g., 200-300, 300-500, or 500-1000 nucleotides in length. Such fragments, whether protein-encoding or not, can be used as probes, primers, and diagnostic reagents. In some embodiments, the isolated nucleic acid molecules encode a full-length zeaxanthin glucosyl transferase, lycopene β-cyclase, geranylgeranyl pyrophosphate synthase, phytoene desaturase, β-carotene hydroxylase, β-carotene C4 oxygenase, or

multifunctional geranylgeranyl pyrophosphate synthase polypeptide. Nucleic acid molecules can be DNA or RNA, linear or circular, and in sense or antisense orientation.

Isolated nucleic acid molecules of the invention can be produced by standard techniques. As used herein, "isolated" refers to a sequence corresponding to part or all of a gene encoding a zeaxanthin glucosyl transferase, lycopene β-cyclase, geranylgeranyl-pyrophosphate synthase, phytoene desaturase, phytoene synthase, β-carotene hydroxylase, β-carotene C4 oxygenase, or multifunctional geranylgeranyl pyrophosphate synthase polypeptide, or an operon encoding two or more such polypeptides, but free of sequences that normally flank one or both sides of the wild-type gene or the operon in a naturally-occurring genome, e.g., a bacterial genome. The term "isolated" as used herein with respect to nucleic acids also includes any non-naturally-occurring nucleic acid sequence since such non-naturally-occurring sequences are not found in nature and do not have immediately contiguous sequences in a naturally-occurring genome.

An isolated nucleic acid can be, for example, a DNA molecule, provided one of the nucleic acid sequences normally found immediately flanking that DNA molecule in a naturally-occurring genome is removed or absent. Thus, an isolated nucleic acid includes, without limitation, a DNA molecule that exists as a separate molecule (e.g., a cDNA or genomic DNA fragment produced by PCR or restriction endonuclease treatment) independent of other sequences as well as recombinant DNA that is incorporated into a vector, an autonomously replicating plasmid, a virus (e.g., a retrovirus, adenovirus, or herpes virus), or into the genomic DNA of a prokaryote or eukaryote. In addition, an isolated nucleic acid can include an engineered nucleic acid such as a recombinant DNA molecule that is part of a hybrid or fusion nucleic acid. A nucleic acid existing among hundreds to millions of other nucleic acids within, for example, cDNA libraries or genomic libraries, or gel slices containing a genomic DNA restriction digest, is not to be considered an isolated nucleic acid.

Isolated nucleic acids within the scope of the invention can be obtained using any method including, without limitation, common molecular cloning and chemical nucleic acid synthesis techniques. For example, polymerase chain reaction (PCR) techniques can be used to obtain an isolated nucleic acid containing a nucleic acid sequence sharing identity with the sequences set forth in SEQ ID NOs: 1, 3, 5, 7, 9, 11, 38, or 44. PCR

refers to a procedure or technique in which target nucleic acids are amplified. Sequence information from the ends of the region of interest or beyond typically is employed to design oligonucleotide primers that are identical in sequence to opposite strands of the template to be amplified. PCR can be used to amplify specific sequences from DNA as well as RNA, including sequences from total genomic DNA or total cellular RNA. Primers are typically 14 to 40 nucleotides in length, but can range from 10 nucleotides to hundreds of nucleotides in length. General PCR techniques are described, for example in PCR Primer: A Laboratory Manual, Ed. by Dieffenbach, C. and Dveksler, G., Cold Spring Harbor Laboratory Press, 1995. When using RNA as a source of template, reverse transcriptase can be used to synthesize complimentary DNA (cDNA) strands.

Isolated nucleic acids of the invention also can be chemically synthesized, either as a single nucleic acid molecule or as a series of oligonucleotides. For example, one or more pairs of long oligonucleotides (e.g., >100 nucleotides) can be synthesized that contain the desired sequence, with each pair containing a short segment of complementary (e.g., about 15 nucleotides) DNA such that a duplex is formed when the oligonucleotide pair is annealed. DNA polymerase is used to extend the oligonucleotides, resulting in a double-stranded nucleic acid molecule per oligonucleotide pair, which then can be ligated into a vector.

Isolated nucleic acids of the invention also can be obtained by mutagenesis. For example, an isolated nucleic acid that shares identity with a sequence set forth in SEQ ID NO: 1, 3, 5, 7, 9, 11, 38, or 44 can be mutated using common molecular cloning techniques (e.g., site-directed mutagenesis). Possible mutations include, without limitation, deletions, insertions, and substitutions, as well as combinations of deletions, insertions, and substitutions. Alignments of nucleic acids of the invention with other known sequences encoding carotenoid enzymes can be used to identify positions to modify. For example, alignment of the nucleotide sequence of SEQ ID NO:5 with other nucleic acids encoding geranyl geranyl pyrophosphate synthases (e.g., from *Erwinia uredovora*) provides guidance as to which nucleotides can be substituted, which nucleotides can be deleted, and at which positions nucleotides can be inserted.

In addition, nucleic acid and amino acid databases (e.g., GenBank®) can be used to obtain an isolated nucleic acid within the scope of the invention. For example, any

nucleic acid sequence having homology to a sequence set forth in SEQ ID NO: 1, 3, 5, 7, 9, 11, 38, or 44, or any amino acid sequence having homology to a sequence set forth in SEQ ID NO: 2, 4, 6, 8, 10, 12, 39, or 45 can be used as a query to search GenBank®.

Furthermore, nucleic acid hybridization techniques can be used to obtain an isolated nucleic acid within the scope of the invention. Briefly, any nucleic acid having some homology to a sequence set forth in SEQ ID NO: 1, 3, 5, 7, 9, 11, 38, or 44 can be used as a probe to identify a similar nucleic acid by hybridization under conditions of moderate to high stringency. Moderately stringent hybridization conditions include hybridization at about 42°C in a hybridization solution containing 25 mM KPO_4 (pH 7.4), 5X SSC, 5X Denhart's solution, 50 μg/mL denatured, sonicated salmon sperm DNA, 50% formamide, 10% Dextran sulfate, and 1-15 ng/mL probe (about $5x10^7$ cpm/μg), and wash steps at about 50°C with a wash solution containing 2X SSC and 0.1% SDS. For high stringency, the same hybridization conditions can be used, but washes are performed at about 65°C with a wash solution containing 0.2X SSC and 0.1% SDS.

Once a nucleic acid is identified, the nucleic acid then can be purified, sequenced, and analyzed to determine whether it is within the scope of the invention as described herein. Hybridization can be done by Southern or Northern analysis to identify a DNA or RNA sequence, respectively, that hybridizes to a probe. The probe can be labeled with biotin, digoxygenin, an enzyme, or a radioisotope such as ^{32}P or ^{35}S. The DNA or RNA to be analyzed can be electrophoretically separated on an agarose or polyacrylamide gel, transferred to nitrocellulose, nylon, or other suitable membrane, and hybridized with the probe using standard techniques well known in the art. See, for example, sections 7.39-7.52 of Sambrook *et al.*, (1989) Molecular Cloning, second edition, Cold Spring harbor Laboratory, Plainview, NY.

Polypeptides

The present invention also features isolated zeaxanthin glucosyl transferase (SEQ ID NO:2), lycopene β-cyclase (SEQ ID NO:4), geranylgeranyl pyrophosphate synthase (SEQ ID NO:6), phytoene desaturase (SEQ ID NO:8), phytoene synthase (SEQ ID NO:10), and β-carotene hydroxylase (SEQ ID NO:12) polypeptides. In addition, the invention features isolated β-carotene C4 oxygenase polypeptides (SEQ ID NO:39) and

multifunctional geranylgeranyl pyrophosphate synthase polypeptides (SEQ ID NO:45). A polypeptide of the invention can have at least 75% sequence identity, e.g., 80%, 85%, 90%, 95%, or 99% sequence identity, to the amino acid sequence of SEQ ID NO:2 or to fragments thereof; at least 83% sequence identity, e.g., 85%, 90%, 95%, or 99% sequence identity, to the amino acid sequence of SEQ ID NO:4 or to fragments thereof; at least 85% sequence identity, e.g., 90%, 95%, or 99% sequence identity, to the amino acid sequence of SEQ ID NO:6 or to fragments thereof; at least 90% sequence identity, e.g., 90%, 92%, 95%, or 99% sequence identity, to the amino acid sequence of SEQ ID NO:8 or to fragments thereof; at least 89% sequence identity, e.g., 90%, 95%, or 99% sequence identity, to the amino acid sequence of SEQ ID NO:10 or to fragments thereof; at least 90% sequence identity, e.g., 95%, or 99% sequence identity, to the amino acid sequence of SEQ ID NO:12 or to fragments thereof; at least 60% sequence identity, e.g., 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 99% sequence identity, to the amino acid sequence of SEQ ID NO:39 or to fragments thereof; or at least 90% sequence identity, e.g., 95% or 99% sequence identity, to the amino acid sequence set forth in SEQ ID NO:45 or to fragments thereof. Percent sequence identity can be determined as described above for nucleic acid molecules.

An "isolated polypeptide" has been separated from cellular components that naturally accompany it. Typically, the polypeptide is isolated when it is at least 60% (e.g., 70%, 80%, 90%, 95%, or 99%), by weight, free from proteins and naturally-occurring organic molecules that are naturally associated with it. In general, an isolated polypeptide will yield a single major band on a non-reducing polyacrylamide gel.

The term "polypeptide" includes any chain of amino acids, regardless of length or post-translational modification. Polypeptides that have identity to the amino acid sequences of SEQ ID NO:2, 4, 6, 8, 10, 12, 39, or 45 can retain the function of the enzyme (see FIG 1 for a schematic of the carotenoid biosynthesis pathway). For example, geranylgeranyl pyrophosphate synthase can produce geranylgeranyl pyrophosphate (GGPP) by condensing together isopentenyl pyrophosphate (IPP) with farnesyl pyrophosphate (FPP). Phytoene synthase can produce phytoene by condensing together two molecules of GGPP. Phytoene desaturase can perform four successive desaturations on phytoene to form lycopene. Lycopene β-cyclase can perform two

successive cyclization reactions on lycopene to form β-carotene. β-carotene hydroxylase can perform two successive hydroxylation reactions on β-carotene to form zeaxanthin. Alternatively, β-carotene hydroxylase can perform two successive hydroxylation reactions on canthaxanthin to form astaxanthin. Zeaxanthin glucosyl transferase can add one or two glucose or other sugar moieties to zeaxanthin to form zeaxanthin monoglycoside or diglycoside, respectively. β-carotene C4 oxygenase can convert the methylene groups at the C4 and C4' positions of the β-carotene or zeaxanthin to form canthaxanthin or astaxanthin, respectively. Multifunctional geranylgeranyl pyrophosphate synthase can directly convert 3 IPP molecules and 1 dimethylallyl pyrophosphate (DMAPP) molecule to 1 GGPP molecule.

In general, conservative amino acid substitutions, i.e., substitutions of similar amino acids, are tolerated without affecting protein function. Similar amino acids are those that are similar in size and/or charge properties. Families of amino acids with similar side chains are known. These families include amino acids with basic side chains (e.g., lysine, arginine, or histidine), acidic side chains (e.g., aspartic acid or glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, or cysteine), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, or tryptophan), β-branched side chains (e.g., threonine, valine, or isoleucine), and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, or histidine).

Mutagenesis also can be used to alter a nucleic acid such that activity of the polypeptide encoded by the nucleic acid is altered (e.g., to increase production of a particular carotenoid). For example, error-prone PCR (e.g., (GeneMorph PCR Mutagenesis Kit; Stratagene Inc. La Jolla, CA; Catalog # 600550; Revision #090001) can be used to mutagenize the *B. aurantiaca crtW* gene (SEQ ID NO:38) to increase the relative amount of di-keto carotenoid (e.g. astaxanthin (3,3'-dihydroxy-β,β-carotene-4,4'-dione) or canthaxanthin (β,β-carotene-4,4'-dione)) relative to mono-keto carotenoid (e.g. echinone (β,β-carotene-4-one) or adonixanthin (3,3'-dihydroxy-β,β-carotene-4-one)) that is produced. In general, the nucleic acid to be mutagenized can be cloned into a vector such as pCR-Blunt II-TOPO (Clontech; Palo Alto, CA) and used as a template for error-prone PCR. For purposes of directed evolution, mutation frequencies of 2-7 nucleotides /

Kbp template (1-4 amino acids mutations / 333 Amino acids) generally are desired. Mutation frequency can be lowered or raised by increasing or decreasing the template concentration, respectively. PCR can be performed according to manufacturer's recommendations. Mutagenized nucleic acid is ligated into an expression vector, which is used to transform a host, and activity of the expressed protein is assessed. For example, in the case of the *crtW* gene, electrocompetent *P. stewartii* (ATCC 8200) cells can be prepared and transformed as described herein, and resulting individual colonies can be screened by visual inspection for a phenotypic change from bright yellow pigmentation (production of zeaxanthin), yellow orange (production of mono-keto carotenoid) or reddish-orange (production of di-keto carotenoid). Production of increased amounts of astaxanthin can be confirmed by HPLC/MS.

Isolated polypeptides of the invention can be obtained, for example, by extraction from a natural source (e.g., a plant or bacteria cell), chemical synthesis, or by recombinant production in a host. For example, a polypeptide of the invention can be produced by ligating a nucleic acid molecule encoding the polypeptide into a nucleic acid construct such as an expression vector, and transforming a bacterial or eukaryotic host cell with the expression vector. In general, nucleic acid constructs include expression control elements operably linked to a nucleic acid sequence encoding a polypeptide of the invention (e.g., zeaxanthin glucosyl transferase, lycopene β-cyclase, geranylgeranyl pyrophosphate synthase, phytoene desaturase, phytoene synthase, β-carotene hydroxylase, β-carotene C4 oxygenase, or multifunctional geranylgeranyl pyrophosphate synthase polypeptides). Expression control elements do not typically encode a gene product, but instead affect the expression of the nucleic acid sequence. As used herein, "operably linked" refers to connection of the expression control elements to the nucleic acid sequence in such a way as to permit expression of the nucleic acid sequence. Expression control elements can include, for example, promoter sequences, enhancer sequences, response elements, polyadenylation sites, or inducible elements. Non-limiting examples of promoters include the *puf* promoter from *Rhodobacter sphaeroides* (GenBank Accession No. E13945), the *nifHDK* promoter from *R. sphaeroides* (GenBank Accession No. AF031817), and the *fliK* promoter from *R. sphaeroides* (GenBank Accession No. U86454).

In bacterial systems, a strain of *E. coli* such as DH10B or BL-21 can be used. Suitable *E. coli* vectors include, but are not limited to, pUC18, pUC19, the pGEX series of vectors that produce fusion proteins with glutathione S-transferase (GST), and pBluescript series of vectors. Transformed *E. coli* are typically grown exponentially then stimulated with isopropylthiogalactopyranoside (IPTG) prior to harvesting. In general, fusion proteins produced from the pGEX series of vectors are soluble and can be purified easily from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites such that the cloned target gene product can be released from the GST moiety.

In eukaryotic host cells, a number of viral-based expression systems can be utilized to express polypeptides of the invention. A nucleic acid encoding a polypeptide of the invention can be cloned into, for example, a baculoviral vector such as pBlueBac (Invitrogen, San Diego, CA) and then used to co-transfect insect cells such as *Spodoptera frugiperda* (Sf9) cells with wild-type DNA from *Autographa californica* multiply enveloped nuclear polyhedrosis virus (AcMNPV). Recombinant viruses producing polypeptides of the invention can be identified by standard methodology. Alternatively, a nucleic acid encoding a polypeptide of the invention can be introduced into a SV40, retroviral, or vaccinia based viral vector and used to infect suitable host cells.

A polypeptide within the scope of the invention can be "engineered" to contain an amino acid sequence that allows the polypeptide to be captured onto an affinity matrix. For example, a tag such as c-myc, hemagglutinin, polyhistidine, or Flag™ tag (Kodak) can be used to aid polypeptide purification. Such tags can be inserted anywhere within the polypeptide including at either the carboxyl or amino termini. Other fusions that could be useful include enzymes that aid in the detection of the polypeptide, such as alkaline phosphatase.

Agrobacterium-mediated transformation, electroporation and particle gun transformation can be used to transform plant cells. Illustrative examples of transformation techniques are described in U.S. Patent No. 5,204,253 (particle gun) and U.S. Patent No. 5,188,958 (*Agrobacterium*). Transformation methods utilizing the Ti and Ri plasmids of *Agrobacterium spp.* typically use binary type vectors. Walkerpeach, C. et

al., in Plant Molecular Biology Manual, S. Gelvin and R. Schilperoort, eds., Kluwer Dordrecht, C1:1-19 (1994). If cell or tissue cultures are used as the recipient tissue for transformation, plants can be regenerated from transformed cultures by techniques known to those skilled in the art.

Engineered cells

Any cell containing an isolated nucleic acid within the scope of the invention is itself within the scope of the invention. This includes, without limitation, prokaryotic cells such as *R. sphaeroides* cells and eukaryotic cells such as plant, yeast, and other fungal cells. It is noted that cells containing an isolated nucleic acid of the invention are not required to express the isolated nucleic acid. In addition, the isolated nucleic acid can be integrated into the genome of the cell or maintained in an episomal state. In other words, cells can be stably or transiently transfected with an isolated nucleic acid of the invention.

Any method can be used to introduce an isolated nucleic acid into a cell. In fact, many methods for introducing nucleic acid into a cell, whether *in vivo* or *in vitro*, are well known to those skilled in the art. For example, calcium phosphate precipitation, conjugation, electroporation, heat shock, lipofection, microinjection, and viral-mediated nucleic acid transfer are common methods that can be used to introduce nucleic acid molecules into a cell. In addition, naked DNA can be delivered directly to cells *in vivo* as describe elsewhere (U.S. Patent Nos. 5,580,859 and 5,589,466). Furthermore, nucleic acid can be introduced into cells by generating transgenic animals.

Any method can be used to identify cells that contain an isolated nucleic acid within the scope of the invention. For example, PCR and nucleic acid hybridization techniques such as Northern and Southern analysis can be used. In some cases, immunohistochemistry and biochemical techniques can be used to determine if a cell contains a particular nucleic acid by detecting the expression of a polypeptide encoded by that particular nucleic acid. For example, the polypeptide of interest can be detected with an antibody having specific binding affinity for that polypeptide, which indicates that that cell not only contains the introduced nucleic acid but also expresses the encoded polypeptide. Enzymatic activities of the polypeptide of interest also can be detected or an

end product (e.g., a particular carotenoid) can be detected as an indication that the cell contains the introduced nucleic acid and expresses the encoded polypeptide from that introduced nucleic acid.

The cells described herein can contain a single copy, or multiple copies (e.g., about 5, 10, 20, 35, 50, 75, 100 or 150 copies), of a particular exogenous nucleic acid. All non-naturally-occurring nucleic acids are considered an exogenous nucleic acid once introduced into the cell. The term "exogenous" as used herein with reference to a nucleic acid and a particular cell refers to any nucleic acid that does not originate from that particular cell as found in nature. Nucleic acid that is naturally-occurring also can be exogenous to a particular cell. For example, an entire operon that is isolated from a bacteria is an exogenous nucleic acid with respect to a second bacteria once that operon is introduced into the second bacteria. For example, a bacterial cell (e.g., *Rhodobacter*) can contain about 50 copies of an exogenous nucleic acid of the invention. In addition, the cells described herein can contain more than one particular exogenous nucleic acid. For example, a bacterial cell can contain about 50 copies of exogenous nucleic acid X as well as about 75 copies of exogenous nucleic acid Y. In these cases, each different nucleic acid can encode a different polypeptide having its own unique enzymatic activity. For example, a bacterial cell can contain two different exogenous nucleic acids such that a high level of astaxanthin or other carotenoid is produced. In addition, a single exogenous nucleic acid can encode one or more polypeptides. For example, a single nucleic acid can contain sequences that encode three or more different polypeptides.

Microorganisms that are suitable for producing carotenoids may or may not naturally produce carotenoids, and include prokaryotic and eukaryotic microorganisms, such as bacteria, yeast, and fungi. In particular, yeast such as *Phaffia rhodozyma* (*Xanthophyllomyces dendrorhous*), *Candida utilis*, and *Saccharomyces cerevisiae*, fungi such as *Neurospora crassa*, *Phycomyces blakesleeanus*, *Blakeslea trispora*, and *Aspergillus sp*, Archaeabacteria such as *Halobacterium salinarium*, and Eubacteria including *Pantoea* species (formerly called *Erwinia*) such as *Pantoea stewartii* (e.g., ATCC Accession #8200), flavobacteria species such as *Xanthobacter autotrophicus* and *Flavobacterium multivorum*, *Zymonomonas mobilis*, *Rhodobacter* species such as *R. sphaeroides* and *R. capsulatus*, *E. coli*, and *E. vulneris* can be used. Other examples of

bacteria that may be used include bacteria in the genus *Sphingomonas* and Gram negative bacteria in the α-subdivision, including, for example, *Paracoccus*, *Azotobacter*, *Agrobacterium*, and *Erythrobacter*. Eubacteria, and especially *R. sphaeroides* and *R. capsulatus*, are particularly useful. *R. sphaeroides* and *R. capsulatus* naturally produce certain carotenoids and grows on defined media. Such *Rhodobacter* species also are non-pyrogenic, minimizing health concerns about use in nutritional supplements. In some embodiments, it can be useful to produce carotenoids in plants and algae such as *Zea mays*, *Brassica napus*, *Lycopersicon esculentum*, *Tagetes erecta*, *Haematococcus pluvialis*, *Dunaliella salina*, *Chlorella protothecoides*, and *Neospongiococcum excentrum*.

It is noted that bacteria can be membranous or non-membranous bacteria. The term "membranous bacteria" as used herein refers to any naturally-occurring, genetically modified, or environmentally modified bacteria having an intracytoplasmic membrane. An intracytoplasmic membrane can be organized in a variety of ways including, without limitation, vesicles, tubules, thylakoid-like membrane sacs, and highly organized membrane stacks. Any method can be used to analyze bacteria for the presence of intracytoplasmic membranes including, without limitation, electron microscopy, light microscopy, and density gradients. See, e.g., Chory et al., (1984) J. Bacteriol., 159:540-554; Niederman and Gibson, Isolation and Physiochemical Properties of Membranes from Purple Photosynthetic Bacteria. In: The Photosynthetic Bacteria, Ed. By Roderick K. Clayton and William R. Sistrom, Plenum Press, pp. 79-118 (1978); and Lueking et al., (1978) J. Biol. Chem., 253: 451-457. Examples of membranous bacteria that can be used include, without limitation, Purple Non-Sulfur Bacteria, including bacteria of the Rhodospirillaceae family such as those in the genus *Rhodobacter* (e.g., *R. sphaeroides* and *R. capsulatus*), the genus *Rhodospirillum*, the genus *Rhodopseudomonas*, the genus *Rhodomicrobium*, and the genus *Rhodophila*. The term "non-membranous bacteria" refers to any bacteria lacking intracytoplasmic membrane. Membranous bacteria can be highly membranous bacteria. The term "highly membranous bacteria" as used herein refers to any bacterium having more intracytoplasmic membrane than *R. sphaeroides* (ATCC 17023) cells have after the *R. sphaeroides* (ATCC 17023) cells have been (1) cultured chemoheterotrophically under aerobic condition for four days, (2) cultured

chemoheterotrophically under anaerobic for four hours, and (3) harvested. Aerobic culture conditions include culturing the cells in the dark at 30°C in the presence of 25% oxygen. Anaerobic culture conditions include culturing the cells in the light at 30°C in the presence of 2% oxygen. After the four hour anaerobic culturing step, the *R. sphaeroides* (ATCC 17023) cells are harvested by centrifugation and analyzed.

Nucleic acids of the invention can be expressed in microorganisms so that detectable amounts of carotenoids are produced. As used herein, "detectable" refers to the ability to detect the carotenoid and any esters or glycosides thereof using standard analytical methodology. In general, carotenoids can be extracted with an organic solvent such as acetone or methanol and detected by an absorption scan from 400-500 nm in the same organic solvent. In some cases, it is desirable to back-extract with a second organic solvent, such as hexane. The maximal absorbance of each carotenoid depends on the solvent that it is in. For example, in acetone, the maximal absorbance of lutein is at 451 nm, while maximal absorbance of zeaxanthin is at 454 nm. In hexane, the maximal absorbance of lutein and zeaxanthin is 446 nm and 450 nm, respectively. High performance liquid chromatography coupled to mass spectrometry also can be used to detect carotenoids. Two reverse phase columns that are connected in series can be used with a solvent gradient of water and acetone. The first column can be a C30 specialty column designed for carotenoid separation (e.g., YMCä Carotenoid S3m; 2.0 x 150 mm, 3mm particle size; Waters Corporation, PN CT99S031502WT) followed by a C8 Xterraä MS column (e.g., Xterraä MS C8; 2.1 x 250 mm, 5mm particle size; Waters Corporation, PN 186000459).

Detectable amounts of carotenoids include 10μg/g dry cell weight (dcw) and greater. For example, about 10 to 100,000μg/g dcw, about 100 to 60,000μg/g dcw, about 500 to 30,000μg/g dcw, about 1000 to 20,000 μg/g dcw, about 5,000 to 55,000 μg/g dcw, or about 30,000 μg/g dcw to about 55,000 μg/g dcw. With respect to algae or other plants or organisms that produce a particular carotenoid, such as astaxanthin, β-carotene, lycopene, or zeaxanthin, "detectable amount" of carotenoid is an amount that is detectable over the endogenous level in the plant or organism.

Depending on the microorganism and the metabolites present within the microorganism, one or more of the following enzymes may be expressed in the

microorganism: geranylgeranyl pyrophosphate synthase, phytoene synthase, phytoene desaturase, lycopene β cyclase, lycopene ε cyclase, zeaxanthin glycosyl transferase, β-carotene hydroxylase, β-carotene C-4 ketolase, and multifunctional geranylgeranyl pyrophosphate synthase. Suitable nucleic acids encoding these enzymes are described above. Also, see, for example, Genbank Accession No. Y15112 for the sequence of carotenoid biosynthesis genes of *Paracoccus marcusii*; Genbank Accession No. D58420 for the carotenoid biosynthesis genes of *Agrobacterium aurantiacum*; Genbank Accession No. M87280 M99707 for the sequence of carotenoid biosynthesis genes of *Erwinia herbicola*; and Genbank Accession No. U62808 for carotenoid biosynthesis genes of *Flavobacterium* sp. Strain R1534.

For example, to produce lycopene in a microorganism that naturally produces neurosporene, such as *Rhodobacter*, an exogenous nucleic acid encoding phytoene desaturase can be expressed, e.g., a phytoene desaturase of the invention, and lycopene can be detected using standard methodology. Expression of additional carotenoid genes in such an engineered cell will allow for production of additional carotenoids. For example, expression of a lycopene β-cyclase in such an engineered cell allows production of detectable amounts of β-carotene, while further expression of a β-carotene hydroxylase allows production of another carotenoid, zeaxanthin. β-carotene and zeaxanthin can be detected using standard methodology and are distinguished by mobility on an HPLC column. Zeaxanthin diglucoside can be produced by further expression of zeaxanthin glucosyl transferase (*crtX*) in an organism that produces zeaxanthin.

Alternatively, canthaxanthin can be produced in organisms that produce phytoene by expression of phytoene desaturase, lycopene β-cyclase, and β-carotene C4 oxygenase, an enzyme that converts the methylene groups at the C4 and C4' positions of the carotenoid to ketone groups. The β-carotene C4 oxygenase from, e.g., *Agrobacterium aurantiacum* or *Haematococcus pluvialis* can be used. See, GenBank Accession Nos. 1136630 and X86782 for a description of the nucleotide and amino acid sequences of the *A. aurantiacum* and *H. pluvialis* enzymes, respectively. The β-carotene C4 oxygenase from *Brevundimonas aurantiaca* also can be used. See, Example 2 for a description of the nucleotide and amino acid sequences. In organisms that do not naturally produce carotenoids, additional enzymes are required for production of canthaxanthin.

Geranylgeranyl pyrophosphate synthase and phytoene synthase can be expressed such that the necessary precursors for canthaxanthin synthesis are present.

Astaxanthin also can be produced in microorganisms that naturally produce carotenoids. For example, a *Rhodobacter* cell can be engineered such that phytoene desaturase, lycopene β-cyclase, β-carotene hydroxylase, and β-carotene C4 oxygenase are expressed and detectable amounts of astaxanthin are produced. Such an organism also can express an enzyme that can modify the 3 or 3' hydroxyl groups of astaxanthin with chemical groups such as glucose (e.g., to produce astaxanthin diglucoside), other sugars, or fatty acids. In addition, a *P. stewartii* cell can be engineered such that β-carotene C4 oxygenase is expressed and detectable amounts of astaxanthin are produced. Astaxanthin can be detected as described above, and has maximal absorbance at 480 nm in acetone.

Yields of astaxanthin and other carotenoids can be increased by expression of a multifunctional geranylgeranyl pyrophosphate synthase, such as that from *S. shibatae* (SEQ ID NO:45) or an Archaebacterial gene from *Archaeoglobus fulgidus* (GenBank Accession No. AF120272), in the engineered microorganism. The archaebacteria GGPPS gene is a homolog of the endogenous *Rhodobacter* gene and encodes an enzyme that directly converts 3 IPP molecules and 1 DMAPP molecule to 1 GGPPS molecule, thereby reducing branching of the carotenoid pathway and eliminating production of other less desirable isoprenoids. Further reductions in less desirable metabolites can be obtained by eliminating endogenous bacteriochlorophyll biosynthesis, which redirects flow into carotenoid biosynthesis. For example, the *bchO*, *bchD*, and *bchI* genes can be deleted and/or replaced with an Archaebacterial GGPPS gene. Additional increases in yield can be obtained by deletion of the endogenous *crtE* gene or the endogenous *crtC*, *crtD*, *crtE*, *crtA*, *crtI*, and *crtF* genes.

Common mutagenesis or knock-out technology can be used to delete endogenous genes. Alternatively, antisense technology can be used to reduce enzymatic activity. For example, a *R. sphaeroides* cell can be engineered to contain a cDNA that encodes an antisense molecule that prevents an enzyme from being made. The term "antisense molecule" as used herein encompasses any nucleic acid that contains sequences that correspond to the coding strand of an endogenous polypeptide. An antisense molecule also can have flanking sequences (e.g., regulatory sequences). Thus, antisense molecules

can be ribozymes or antisense oligonucleotides. A ribozyme can have any general structure including, without limitation, hairpin, hammerhead, or axhead structures, provided the molecule cleaves RNA.

Control of the Ratio of Carotenoids

The amount of particular carotenoids, such as astaxanthin to canthaxanthin, or astaxanthin to zeaxanthin, can be controlled by expression of carotenoid genes from an inducible promoter or by use of constitutive promoters of different strengths. As used herein, "inducible" refers to both up-regulation and down regulation. An inducible promoter is a promoter that is capable of directly or indirectly activating transcription of one or more DNA sequences or genes in response to an inducer. In the absence of an inducer, the DNA sequences or genes will not be transcribed. The inducer can be a chemical agent such as a protein, metabolite, growth regulator, phenolic compound, or a physiological stress imposed directly by heat, cold, salt, or toxic elements, or indirectly through the action of a pathogen or disease agent such as a virus. The inducer also can be an illumination agent such as light, darkness and light's various aspects, which include wavelength, intensity, fluorescence, direction, and duration. Examples of inducible promoters include the lac system and the tetracycline resistance system from *E. coli*. In one version of the lac system, expression of lac operator-linked sequences is constitutively activated by a lacR-VP16 fusion protein and is turned off in the presence of IPTG. In another version of the lac system, a lacR-VP16 variant is used that binds to lac operators in the presence of IPTG, which can be enhanced by increasing the temperature of the cells.

Components of the tetracycline (Tc) resistance system also can be used to regulate gene expression. For example, the Tet repressor (TetR), which binds to tet operator sequences in the absence of tetracycline and represses gene transcription, can be used to repress transcription from a promoter containing tet operator sequences. TetR also can be fused to the activation domain of VP 16 to create a tetracycline-controlled transcriptional activator (tTA), which is regulated by tetracycline in the same manner as TetR, i.e., tTA binds to tet operator sequences in the absence of tetracycline but not in the presence of

tetracycline. Thus, in this system, in the continuous presence of Tc, gene expression is repressed, and to induce transcription, Tc is removed.

Alternative methods of controlling the ratio of carotenoids include using enzyme inhibitors to regulate the activity levels of particular enzymes.

Production of Carotenoids

Carotenoids can be produced *in vitro* or *in vivo*. For example, one or more polypeptides of the invention can be contacted with an appropriate substrate or combination of substrates to produce the desired carotenoid (e.g., astaxanthin). See, FIG. 1 for a schematic of the carotenoid biosynthetic pathway.

A particular carotenoid (e.g., astaxanthin, lycopene, β-carotene, lutein, zeaxanthin, zeaxanthin diglucoside, or canthaxanthin) also can be produced by providing an engineered microorganism and culturing the provided microorganism with culture medium such that the carotenoid is produced. In general, the culture media and/or culture conditions are such that the microorganisms grow to an adequate density and produce the desired compound efficiently. For large-scale production processes, the following methods can be used. First, a large tank (e.g., a 100 gallon, 200 gallon, 500 gallon, or more tank) containing appropriate culture medium with, for example, a glucose carbon source is inoculated with a particular microorganism. After inoculation, the microorganisms are incubated to allow biomass to be produced. Once a desired biomass is reached, the broth containing the microorganisms can be transferred to a second tank. This second tank can be any size. For example, the second tank can be larger, smaller, or the same size as the first tank. Typically, the second tank is larger than the first such that additional culture medium can be added to the broth from the first tank. In addition, the culture medium within this second tank can be the same as, or different from, that used in the first tank. For example, the first tank can contain medium with xylose, while the second tank contains medium with glucose.

Once transferred, the microorganisms can be incubated to allow for the production of the desired carotenoid. Once produced, any method can be used to isolate the desired compound. For example, if the microorganism releases the desired carotenoid into the broth, then common separation techniques can be used to remove the biomass

from the broth, and common isolation procedures (e.g., extraction, distillation, and ion-exchange procedures) can be used to obtain the carotenoid from the microorganism-free broth. In addition, the desired carotenoid can be isolated while it is being produced, or it can be isolated from the broth after the product production phase has been terminated. If the microorganism retains the desired carotenoid, the biomass can be collected and the carotenoid can be released by treating the biomass or the carotenoid can be extracted directly from the biomass. Extracted carotenoid can be formulated as a nutraceutical. As used herein, a nutraceutical refers to a compound(s) that can be incorporated into a food, tablet, powder, or other medicinal form that, upon ingestion by a subject, provides a specific medical or physiological benefit to the subject.

Alternatively, the biomass can be collected and dried, without extracting the carotenoids. The biomass then can be formulated for human consumption (e.g., as a dietary supplement) or as an animal feed (e.g., for companion animals such as dogs, cats, and horses, or for production animals). For example, the biomass can be formulated for consumption by poultry such as chickens and turkeys, or by cattle, pigs, and sheep. Feeding of such compositions may increase yield of breast meat in poultry and may increase weight gain in other farm animals. In addition, the carotenoids may increase shelf-life of meat products due to the increased antioxidant protection afforded by the carotenoids. The biomass also can be formulated for use in aquaculture. For example, biomass that includes an engineered microorganism that is producing, e.g., astaxanthin and/or canthaxanthin, can be fed to fish or crustaceans to pigment the flesh or carapace, respectively. Such a composition is particularly useful for feeding to fish such as salmon, trout, sea breem, or snapper, or crustaceans such as shrimp, lobster, and crab.

One or more components can be added to the biomass before or after drying, including vitamins, other carotenoids, antioxidants such as ethoxyquin, vitamin E, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), or ascorbyl palmitate, vegetable oils such as corn oil, safflower oil, sunflower oil, or soybean oil, and an edible emulsifier, such as soy bean lecithin or sorbitan esters. Addition of antioxidants and vegetable oils can help prevent degradation of the carotenoid during processing (e.g., drying), shipment, and storage of the composition.

The invention will be further described in the following examples, which do not limit the scope of the invention described in the claims.

EXAMPLES

Example 1 - Cloning of the zeaxanthin gene cluster from *Pantoea stewartii*:

Genomic DNA from *P. stewartii* was isolated and digested with restriction enzymes to yield genomic DNA fragments approximately 8-10 kB in size. These genomic DNA fragments were ligated into a vector cut with the same restriction enzyme, and electroporated into electrocompetent *E. coli*. Transformant colonies were individually picked and transferred onto fresh solid media with the appropriate antibiotic selection (ampicillin/ampicillin substitute). It was thought that *E. coli* colonies containing the *P. stewartii* carotenoid genes would appear yellow in color due to the production of zeaxanthin pigment or red due to the production of lycopene. Although at least 2000 ampicillin resistant *E. coli* transformants were screened, none of the colonies were found to contain the *P. stewartii* carotenoid genes.

Instead, a second, PCR based method was used to identify and sequence the carotenoid (crt) gene cluster from *P. stewartii* genomic DNA. Degenerate primers were designed based on homologous regions identified in the crt genes from *Erwinia herbicola* and *Erwinia uredovora*. Table 2 provides the position of the crt genes in *E. herbicola* and *E. uredovora*.

TABLE 2

Position of crt genes in *E. herbicola* and *E. uredovora*

Gene name	Start of Gene (nucleotide #)		End of Gene (nucleotide #)	
	E. herbicola	*E. uredovora*	*E. herbicola*	*E. uredovora*
CrtE	3535	198	4458	1133
Orf-6	4521		5564	
CrtX	5561	1143	6802	2438
CrtY	6799	2422	7959	3570
CrtI	7956	3582	9434	5060
CrtB	9431	5096	10360	5986
CrtZ	10826 (complement)	6452 (complement)	10296 complement	5925 (complement)
Orf-12	12127 complement		10916 complement	

The following primers were designed (Table 3) and used in various combinations to yield PCR products of varying lengths. *P. stewartii* genomic DNA was used as template.

TABLE 3
Sequences of Degenerate Primers

Primer Name	Primer Sequence	SEQ ID NO
P.s.BCHy1	5'-ATYATGCACGGCTGGGGWTGGSGMTGGCA - 3'	13
P.s. BCHy2	5' - GGCCARCGYTGATGCACCAGMCCGTCRTGCA - 3'	14
P.s.PS1	5' - CTGATGCTCTAYGCCTGGTGCCGCCA - 3'	15
P.s.PS2	5' - TCGCGRGCRATRTTSGTCARCTG - 3'	16
P.s.LBC1	5' - ATBMTSATGGAYGCSACSGT - 3'	17
P.s.LBC2	5' - YTRATCGARGAYACGCRCTA - 3'	18
P.s.LBC3	5' - RSGGCAGYGAATAGCCRGTG - 3'	19
P.s.LBC4	5' - AACAGCATSCGRTTCAGCAKGCGSA - 3'	20
P.s.PD5	5' - CCGACGGTKATCACCGATCC - 3'	21
P.s.PD6	5' – CTGCGCCSACCAGGTAGAG - 3'	22
P.sGGPPS1	5' - CTYGACGAYATGCCCTGCATGGAC – 3' (MD92)	23
P.s.GGPPS2	5' - GTCGATTTWCCSGCGTCCTKATTG - 3' (MD93)	24

PCR was performed in a Gradient Thermocycler, and was started by incubating at 96°C for 5 minutes, followed by 40 cycles of denaturation at 96°C for 30 seconds, annealing at 40°C/45°C/50°C/55°C/or 60°C for 105 seconds, and extension at 72°C for 90 seconds, followed by incubation at 72°C for 10 mins. The concentration of $MgCl_2$ in the PCR reactions also was varied and ranged from a final concentration of 1.5 mM to 6 mM. Table 4 provides the predicted size of the PCR products with various primer combinations.

TABLE 4
Expected sizes of PCR Products

Primer Combination	PCR product length (bp)	Product Observed
BCHy1/BCHy2	230	Yes
PS1/PS1	410	Yes
LBC1/LBC3	320	Yes
LBC1/LBC4	460	Yes
PD1/PD2	420	No
PD1/PD4	1260	No
LBC2/LBC3	240	No
PD3/PD4	410	Yes
LBC2/LBC4	380	Yes
PD5/PD6	1200	Yes
PS1/PS2	410	Yes
BCHy1/BCHy2	230	Yes
PsGGPPS1/PsGGPPS2	470	Yes
LBCDown1/PDUp1	470	Yes
PDDown1/PSUp1	300	Yes
BCHyDown1/PSDown1	700	Yes
LBCUp1/GGPPSdn1	1600	Yes

PCR reactions were electrophoresed through agarose gels to estimate sizes of PCR products and DNA was extracted from the gel using a Qiagen gel extraction kit. The purified PCR products were submitted to the Advanced Genetic Analysis Center (AGAC) at the University of Minnesota for sequencing. The obtained DNA sequences were subjected to BLAST analysis to determine if the sequences were homologous to *crt* genes from other bacteria. Sequence analysis of the 1.2-kb DNA fragment indicated that there was homology to phytoene desaturase (*crtI*) genes from *E. herbicola* and *E. uredovora*, while the 0.47 kB product had homology with the *crtE* genes from *E. herbicola* and *E. uredovora*.

Based on the DNA sequence information generated using the degenerate primers and amplified regions of the carotenoid genes from *P. stewartii*, primers specific for the *P. stewartii crt* genes were designed and are shown in Table 5. These specific primers were used to obtain information upstream and downstream of the DNA regions amplified

with the degenerate primers. This rationale was used to extend and obtain DNA sequence information about the *P. stewartii crt* genes.

TABLE 5
***P. stewartii* primers**

Primer	Sequence	SEQ ID NO
PsOp.crtE	5'-GGCCGAATTCCAACGATGCTCTGGCAGTTA-3'	25
PSOp.crtZ(-)	5'-GGCCAGATCTACTTCAGGCGACGCTGAGAG-3'	26
PsOp.crtZ(+)	5'-GGCCAGATCTTACGCGCGGGTAAAGCCAAT-3'	27
PsOp.crtZ(2+)	5'-GGCCTCTAGAATTACCGCGTGGTTCTGAAG-3'	28
PsOp.crtZ(2-)	5'-GGCCTCTAGATCTGTACGCGCCACCGTTAT-3'	29

After unsuccessful attempts at completing the sequence *crt* gene cluster sequence from *P. stewartii* using PCR, the Universal Genome Walker kit from Clontech was used to obtain the complete the sequence of the *P. stewartii crtE* and *crtZ* genes. This kit uses a PCR based approach. The following primer pairs were synthesized and used for the genome walking experiments: GWcrtE2, 5' - CATCGGTAAGATCGTCAAGCAACTGAA - 3' (SEQ ID NO:30) and GWcrtE1, 5' - GATTTACCTGCATCCTGATTGATGTCT - 3' (SEQ ID NO:31); and GWcrtZ1, 5' - ATGTATAACCGTTTCAGGTAGCCTTTG - 3' (SEQ ID NO:32) and GWcrtZ2, 5' - AATACAGTAAACCATAAGCGGTCATGC - 3' (SEQ ID NO:33). The sequences of the *crt* genes and encoded proteins from *P. stewartii* were compared to the sequence of the crt genes and proteins from *E. herbicola* and *E. uredovora* using BLAST under default parameters. See, SEQ ID NOS 1-12 for the nucleotide and amino acid sequences of the *P. stewartii crt* genes. The results of the alignment are provided in Table 6.

TABLE 6
Comparison of crt genes and proteins from *P. stewartii* to *E. herbicola* and *E. uredovora*

	Comparison of nucleotide sequence of *P. stewartii* to		Comparison of protein sequence of *P. stewartii* to	
Gene	*E. herbicola*	*E. uredovora*	*E. herbicola*	*E. uredovora*
crtE	59%	80%	81%	83%
crtX	56%	75%	75%	74%
crtY	58%	77%	83%	82%

	Comparison of nucleotide sequence of *P. stewartii* to		Comparison of protein sequence of *P. stewartii* to	
Gene	*E. herbicola*	*E. uredovora*	*E. herbicola*	*E. uredovora*
crtI	69%	81%	89%	89%
crtB	63%	81%	88%	88%
crtZ	65%	84%	65%	88%

Example 2 - Cloning of a β-carotene C4 Oxygenase from *Brevundimonas aurantiaca*: Degenerate PCR primers for *crtW* were designed based on *crtW* genes from *Bradyrhizobium*, *Alcaligenes*, *Agrobacterium aurantiacum*, and *Paracoccus marcusii*. The primers had the following sequences: (*crtW*(181P.m.) - 5'TTCATCATCGCGCATGAC3' (SEQ ID NO:34) and *crtW*(668P.m.)- 5'AGRTGRTGYTCGTGRTGA (SEQ ID NO:35), and were synthesized by Integrated DNA Technologies Inc. (Coralville, IA). PCR was performed in a mastercycler gradient machine (Eppendorf) with genomic DNA from *B. aurantiaca* (ATCC Accession No. 15266). Reaction conditions included five minutes at 96°C, followed by 30 cycles of denaturation at 94°C for 30 sec., annealing at 50°C for 2 min., and extension at 72°C for 2 min 30 sec, and a final 72°C incubation for 10 min. An approximately 500-bp PCR product was obtained and cloned into the vector pCR-BluntII-TOPO (Invitrogen Corp. Carlsbad, CA).

Independent clones were sequenced using the universal M13 forward and reverse primers. DNA sequencing was carried out at AGAC, University of Minnesota, St. Paul, MN. Partial nucleotide sequence of the *crtW* gene was obtained. Alignment of the partial sequence with known *crtW* genes indicated that the sequences aligned toward the N-terminus and C-terminus, respectively, of the *crtW* genes from *Bradyrhizobium*, *Alcaligenes*, *Agrobacterium aurantiacum*, and *Paracoccus marcusii*. The Universal Genome Walker kit from Clontech was used to obtain the complete the sequence of the *B. aurantiaca crtW* gene. Primers were synthesized based on the partial sequence and used for the genome walking experiments.

Upon obtaining sequence from the ends of the gene, the following oligonucleotide primers were synthesized and used to amplify the complete *crtW* gene from genomic DNA: 5'-GCGGCATAGGCTAGATTGAAG-3' (primer 1, Tm = 72°C, SEQ ID NO:36) and 5'-GCGAGTTCCTTCTCACCTAT-3' (primer 2, Tm = 67°C, SEQ ID NO:37). *B.*

aurantiaca (ATCC 15266) genomic DNA was prepared with the Qiagen genomic-tip 500G kit (Valencia, CA; Catalog # 10262) following the manufacturers protocol. Briefly, 30 ml of *B. aurantiaca* culture were grown overnight at 30°C in ATCC medium 36 (Caulobacter medium; 2g/l peptone, 1 g/l yeast extract, 0.2 g/l MgSO4.7H20). Cultures were harvested by centrifugation (15,000 x g; 10 minutes) and genomic DNA purified following the manufacturer's recommended protocol (Qiagen Genomic DNA Handbook for Blood, Cultured Cells, Tissue, Mouse Tails, Yeast, Bacteria (Gram- & some Gram+). The Expand DNA polymerase system (Roche Molecular Biochemicals, Indianapolis, IN; catalog # 1732641) was used in a reaction that included 2 µl of *B. aurantiaca* genomic DNA (50 ng/µl), 1 µl of primer 1 (100 pmol/µl), 1 µl of primer 2 (100 pmol/µl), 5 µl of 10x PCR buffer, 1 µl of Expand DNA polymerase (3.5 U/µl), 2.5 µl of dimethyl sulfoxide (DMSO), 2 µl of dNTP's (10 nmol/µl each), and 35.5 µl of dd H_2O. Reaction conditions included five minutes at 96°C, followed by 30 cycles of denaturation at 94°C for 30 sec., annealing at 50°C for 2 min., and extension at 72°C for 2 min 30 sec, and a final 72°C incubation for 10 min.

PCR products were electrophoresed through a 0.8% agarose gel and the ~0.85 kB band was excised from the gel and purified using the Qiagen QIAquick Gel Extraction Kit (catalog #28704) following the manufacturer's recommended protocol (QIAquick Spin Handbook). Gel-purified PCR product was cloned into the blunt-end cloning site of pCR-Blunt II-TOPO (Clontech; Palo Alto, CA) to generate pTOPOcrtW. Ligation mixtures were electroporated (25 µF, 200 Ohms, 12.5 KV/cm) into *E. coli* DH10B electromax cells (Gibco BRL; Gaithersburg, MD; catalog #18290-015). Transformants were allowed to recover 60 minutes at 37°C with shaking in 1 ml of SOC medium. Cells were plated on LB agar + 50 µg/ml kanamycin and allowed to grow overnight at 37°C. Transformant colonies were inoculated into 1 ml LB broth + 50 µg/ml kanamycin and allowed to grow overnight at 37°C with shaking. Minipreps were prepared using the QIAprep Spin Miniprep Kit (50) (catalog #27104) following the manufacturer's protocol and the presence of pTOPOcrtW was screened for by restriction analysis with *Eco*RI. *Eco*RI digests of pTOPOcrtW yielded products of ~0.85 Kbp and 3.5 Kbp.

The *crtW* gene was sequenced by AGAC, University of Minnesota, St. Paul, MN. The nucleotide sequence of the *crtW* gene from *B. aurantiaca* is provided in SEQ ID NO:38, and the protein encoded by the *crtW* gene is provided in SEQ ID NO:39.

Example 3 - Transformation of pTOPOcrtW into *Pantoea stewartii* and production of astaxanthin and adonixanthin in *P.stewartii*::pTOPOcrtW: The following protocol describes expression of *crtW* in the zeaxanthin producing host *P. stewartii*. This yields a transformed host that is capable of producing astaxanthin (i.e., 3,3'-dihydroxy-β,β-carotene-4,4'-dione) and adonixanthin (3,3'-dihydroxy-β,β-carotene-4-one). Electrocompetent *P. stewartii* (ATCC 8200) cells were prepared by culturing 50 ml of a 5% inoculum of *P. stewartii* cells in LB at 30°C -with agitation (250 rpm) until an OD_{590} of 0.5-1.0 was reached. The bacteria were washed in 50 ml of 10mM HEPES (pH 7.0) and centrifuged for 10 minutes at 10,000xg. The wash was repeated with 25 ml of 10mM HEPES (pH 7.0) followed by the same centrifugation protocol. The cells then were washed once in 25 ml of 10% glycerol. Following centrifugation, the cells were resuspended in 500 µl of 10% glycerol. Forty µl aliquots were frozen and kept at –80°C until use.

Plasmid TOPOcrtW was electroporated into electrocompetent *P. stewartii* cells (25 µF, 25 KV/cm, 200 Ohms) and plated onto LB agar plates containing 50 µg/ml kanamycin. As a negative control, pCR-Blunt II-TOPO self-ligated parental vector also was electroporated into *P. stewartii* and plated onto LB agar plates containing 50 µg/ml kanamycin. Individual colonies of *P. stewartii*::pTOPOcrtW were screened by visual inspection for a phenotypic change from bright yellow pigmentation (production of zeaxanthin) to a reddish-orange pigmentation (production of astaxanthin) and chosen for further pigment analysis. No phenotypic change was noted for individual colonies of *P. stewartii*:: pCR-Blunt II-TOPO, so clones were randomly chosen for pigment analysis.

Production of astaxanthin was confirmed by HPLC/MS. Carotenoids were extracted from cells harvested from 5 day old cultures of *P. stewartii*::pTOPOcrtW or *P. stewartii*:: pCR-Blunt II-TOPO (25 ml) grown in LB with 50 µg/ml kanamycin by resuspending the washed cell pellet in 5 ml of acetone. Glass beads were added and the mixture was incubated for 60 minutes at room temperature in the dark with occasional

vortexing. The cells were separated from the acetone extract by centrifugation at 15,000 x g for 10 minutes. The acetone supernatant then was analyzed by HPLC/MS.

A Waters 2790 LC system was used with two reverse-phase C30 specialty columns designed for carotenoid separation (YMCa Carotenoid S3m; 2.0 X 150 mm, 3 mm particle size; Waters Corporation, PN CT99S031502WT)), in tandem. The columns were run at room temperature. A gradient of Mobile Phase A (0.1% acetic acid) and Mobile Phase B (90% acetone) was used to separate zeaxanthin and astaxanthin according to the following gradient timetable: 0 min (10%A, 90%B), 10 min (100%B), 12 min (10%A, 90%B), 15 min (10%A, 90%B). Flow rate was 0.3 ml/min. Samples were stored at 20°C in an autosampler and a volume of 25 µL was injected. A Waters 996 Photodiode array detector, 350-550 nm, was used to detect zeaxanthin and astaxanthin. Under these chromatography conditions astaxanthin eluted at approximately 5.42-5.51 min and zeaxanthin eluted at approximately 6.22-6.4 min.

Carotenoid standards were used to identify the peaks. Astaxanthin was obtained from Sigma Chemical Co. (St. Louis, MO) and zeaxanthin was obtained from Extrasynthese (France). UV-Vis absorbtion spectra were used as diagnostic features for the carotenoids as were the molecular ion and fragmentation patterns generated using mass spectrometry. A positive-ion atmospheric pressure chemical ionization mass spectrometer was used; scan range, 400-800 m/z with a quadripole ion trap. A representative HPLC chromatogram is shown in FIG 3, which confirms production of astaxanthin in *P. stewartii* transformed with the *B. aurantiaca crtW* gene.

Example 4 - Simultaneous Production of CoQ-10 and (3S, 3'S) Astaxanthin in a Microorganism: Although *Phaffia rhodozyma* is not capable of producing the 3S, 3'S isoform of astaxanthin, it is known to produce Coenzyme Q-10. This compound has been found to have particularly high value as a nutraceutical. The current invention is of particular value since *R. sphaeroides* is known to produce Coenzyme Q-10 and has been transformed with genes that, while novel, are nevertheless homologous to native genes in the MABP. Consequently, the described organism can be expected to simultaneously produce both Coenzyme Q-10 and (3S, 3'S)-ATX. This is the first described production of the production of both (3S, 3'S)-ATX and Coenzyme Q-10 in a single microbial host.

The identification of (3S, 3'S)-ATX can be accomplished as described by Maoka, T., et al. J. Chromatogr. 318:122-124 (1985). Briefly, this consists of extraction of the carotenoid pigments by contacting the biomass with a suitable organic solvent such as actetone or dichloromethane. The carotenoid extract is then dried under a stream of liquid nitrogen and resuspended in a solvent of n-hexane-dichloromethane-ethanol (48:16:0.6). The extract is applied to a Sumipax OA-2000 (particle size 10uM) 250 x 4 mm I.D. (Sumitomo Chemicals, Osaka, Japan) chiral resolution HPLC column at a flow rate of 0.8 ml/min. Generally, the order of elution is expected to be (3R, 3'R)-ATX followed by (3R, 3'S; 3S, 3'R)-ATX followed by (3S, 3'S)-ATX. A similar separation is described in Maoka, T., et al. Comp. Biochem. Physiol. 83B:121-124 (1986). Briefly, this consists of isolation of the carotenoid, derivitization to the dibenzoate form with benzoyl chloride and separation of the enantiomers using a Sumipax OA-2000 chiral resolution HPLC column.

Example 5 – Transformation of the multifunctional GGPP synthase from *Archeoglobus fulgidus* into *Rhodobacter* strain ppsr- with the *crtY* and *crtI* genes from *Pantoea stewartii* inserted into the chromosome: The following protocol describes the generation of a β-carotene producing strain of *R. sphaeroides* (ATCC 35053), a facultative photoheterotroph, in which the *ppsr* gene was deleted by using the in-frame deletion procedure of Higuchi, R., et al, Nucleic Acid Res. 16: 7351-7367 to generate strain ΔREG. Table 7 describes the strains and plasmids used in this example. PpsR is a transcription factor that is involved in the repression of photosysem gene expression under aerobic growth conditions. The region of the chromosome that included the native *tspO*, *crtC*, *crtD*, *crtE* and *crtF* genes of ΔREG were replaced by the lycopene β cyclase (*crtY*) and phytoene desaturase (*crtI*) genes from *P. stewartii* using the procedure of Oh and Kaplan, Biochemistry 38:2688-2696 (1999); and Lenz, et al., J. Bacteriology 176:4385-4393 (1994), to generate the strain ΔREG(Δ5:YI). Briefly, the *crtY* and *crt I* genes were cloned into pLO1, a suicide vector for *R. sphaeroides* containing the Kanamycin resistance gene and the *Bacillus subtilis sacB* gene encoding sensitivity to sucrose. DNA fragments flanking the *crtYI* genes and identical in sequence to ~500 bp internal fragments of the *R. sphaeroides tspO* and *crtF* genes were then cloned

into pLO1. These flanking DNA regions correspond to the desired region for insertion of the *crtYI* genes. Insertion of the *crtYI* genes in ΔREG was confirmed using PCR analyses and appropriate PCR primers specific to the *crtYI* genes as well as flanking regions of the *R.sphaeroides* genome. The *crtYI* (*P. stewartii*) insertion and *tspO, crtC, crtD, crtE* and *crtF* (*R. sphaeroides*) deletion resulted in the lack of native carotenoid production and a change in the pigmentation from red to green, confirming the insertion event.

TABLE 7

Description of *Rhodobacter* Strains and Plasmids

Strain	Description	Major Carotenoid Produced	Comments
ΔREG	ATCC 35053; *ppsR* regulatory mutant	Sphaeroidenone (Native Carotenoid)	Regulatory mutant
ΔREG(Δ5:YI)	*CrtY* and *crtI* genes of *P. stewartii* replaced 5 host genes (*tspO*, *crtC*, *crtD*, *crtE* and *crtF*) on chromosome	None	β-carotene biosynthetic genes placed in chromosome. No carotenoid production because of *crtE* deletion
ΔREG(Δ5:YI)::pPctrl	Control vector introduced into ΔREG(Δ5:YI) host	None	Control vector contains *rrnB* promoter but no biosynthetic genes
ΔREG(Δ5:YI)::pPgps	*gps* gene of *A. fulgidus* inserted into pPctrl control vector and introduced into ΔREG(Δ5:YI) host	β-Carotene	*gps* gene on plasmid complements *crtE* deletion. Complete pathway for β-carotene production

Strain	Description	Major Carotenoid Produced	Comments
ΔREG(Δ5:YI) (ΔA:gps)	*gps* gene of *A. fulgidus* replaced *crtA* host gene on chromosome of ΔREG(Δ5:YI) host	β-Carotene	*gps* gene inserted into genome complements *crtE* deletion. Complete pathway for β-carotene production
ΔREG(Δ5:YI) (ΔA:gps) ::pPWZ	*crtW* and *crtZ* genes inserted into pPctrl control vector and introduced into ΔREG(Δ5:YI) (ΔA:gps) host	Astaxanthin	*crtW* and *crtZ* genes convert β-carotene into astaxanthin
ΔREG(Δ5:YI) (ΔA:gps) ::pPgpsWZ	*gps, crtW* and *crtZ* genes inserted into pPctrl control vector and introduced into ΔREG(Δ5:YI) (ΔA:gps) host	Astaxanthin	Additional copies of *A. fulgidus gps* gene on plasmid increases production of astaxanthin

Plasmids	Genetic elements inserted
PBBR1MCS2	None
PPctrl	*rrnB* promoter
PPgps	*rrnB* promoter, *A. fulgidus gps*
PPWZ	*rrnB* promoter, *P. stewartii crtZ*, *B. aurantiacum crtW*
PPgpsWZ	*rrnB* promoter, *A. fulgidus gps* *P. stewartii crtZ*, *B. aurantiacum crtW*

The pPctrl vector was constructed by inserting a copy of the *R. sphaeroides rrnB* promoter (GenBank Accession # X53854; rrnBP) into the vector pBBR1MCS2 (GenBank Accession # U23751). The *rrnB* promoter was isolated from the vector pTEX24 (S. Kaplan) by a *Bam*HI restriction enzyme digest, which released the promoter as a 363 bp fragment. This fragment was gel purified from a 2% Tris-acetate-EDTA (TAE) agarose gel. To prepare the pBBR1MCS2 vector for ligation, it also was digested with *Bam*HI

and the enzyme heat inactivated at 80°C for 20 minutes. The digested vector was dephosphorylated with shrimp alkaline phosphatase (Roche Molecular Biochemicals, Indianapolis, IN), and gel purified from a 1% TAE-agarose gel. The prepared vector and the *rrnB* fragment were ligated using T4 DNA ligase at 16°C for 16 hours to generate the plasmid pPctrl. One μL of ligation reaction was used to electroporate 40 μL of *E. coli* ElectromaxTM DH10BTM cells (Life Technologies, Inc., Rockville, MD).

Electroporated cells were plated on LB media containing 25 μg/mL of kanamycin (LBK). pPctrl DNA was isolated from cultures of single colonies and was digested with *Hind* III to confirm the presence of a single insertion of the *rrnB* promoter. The sequence of pPctrl also was confirmed by DNA sequencing.

The multifunctional GGPP synthase (*gps*) gene from *A. fulgidus* (GenBank Accession No. AF120272) was cloned into the multiple cloning site of pPctrl to generate the construct pPgps.

Electrocompetent ΔREG(Δ5:YI) cells were prepared as follows: 5 ml cultures were inoculated using Sistrom's media supplemented with trace elements, vitamins (O'Gara, et al., J. Bacteriol. 180:4044-4050 (1988); Cohen-Bazire, et al. J. Cell. Comp. Physiol. 49:25-68 (1957)) and 0.4% glucose as a carbon source, and grown overnight at 30°C with shaking. This culture was diluted 1/100 in 300 mL of the same media and grown to an OD_{660} of 0.5-0.8. The cells were chilled on ice for 10 minutes and then centrifuged for 6 minutes at 7,500 g. The supernatant was discarded and the cell pellet was resuspended in ice-cold 10% glycerol at half of the original volume. The cells were pelleted by centrifugation for 6 minutes at 7,500 g. The supernatant was again discarded and cells were resuspended in ice cold 10% glycerol at one quarter of the original volume. The last centrifugation and resuspension steps were repeated, followed by centrifugation for 6 minutes at 7,500 g. The supernatant was decanted and the cells resuspended in the small volume of glycerol that did not drain out. Additional ice-cold 10% glycerol was added to resuspend the cells if necessary. Forty μL of the resuspended cells was used in a test electroporation (see below) to determine if the cells needed to be concentrated by centrifugation or diluted with 10% ice-cold glycerol. Time constants of 8.5-9.0 resulted in good transformation efficiencies. Once an acceptable time constant was achieved, cells

were aliquoted into cold microfuge tubes and stored at -80°C. All water used for media and glycerol was 18 Mohm or higher.

Electroporation of ΔREG(Δ5:YI) was carried out as follows. One μL of pPgps or pPctrl vector DNA was gently mixed into 40 μL of ΔREG(Δ5:YI) electrocompetent cells, which then were transferred to an electroporation cuvette with a 0.2 cM electrode gap. Electroporations were conducted using a Biorad Gene Pulser II (Biorad, Hercules, CA) with settings at 2.5 kV of potential, 400 ohms of resistance, and 25 μF of capacitance. Cells were recovered in 400 μL SOC media at 30°C for 6-16 hours. The cells were then plated, 200 μL per plate, on LB medium containing 50 μg/ml kanamycin and incubated at 30°C for 5-6 days.

After incubation, greenish colonies were observed on plates of ΔREG(Δ5:YI) transformed with pPctrl plasmid DNA. The colonies that appeared on plates of ΔREG(Δ5:YI) transformed with pPgps plasmid DNA appeared yellow. The yellow pigmentation was indicative of β-carotene production in ΔREG(Δ5:YI) expressing the *A. fulgidus gps* gene from pPgps.

Single yellow colonies were grown up in Sistrom's liquid media supplemented with vitamins, trace elements and 0.4% glucose as well as 50 μg/ml kanamycin, at 30°C with shaking for 24-48 hours. Carotenoids were extracted and subjected to LCMS analysis as described above. Under the chromatography conditions used, β-carotene eluted at approximately 13.87-14.2 min. β-carotene standard (Sigma chemical, St. Louis, MO) was used to identify the peaks. The UV-Vis absorption spectra and the retention time using HPLC were used as diagnostic features for β-carotene identification in ΔREG(Δ5:YI) transformed with pPgps DNA, as well as the molecular ion and fragmentation patterns generated during mass spectrometry. Thus, the production of β-carotene was confirmed in ΔREG(Δ5:YI) expressing the *A. fulgidus gps* gene from pPgps.

Example 6 – Transformation of the β-carotene C-4 ketolase (*crtW*) gene from *Brevumdimonas aurantiacum* and β-carotene hydroxylase (*crtZ*) from *P. stewartii* into the ΔREG(Δ5:Y1) strain of *Rhodobacter* with the *gps* gene from *Archeoglobus fulgidus* inserted into the chromosome: The following protocol describes the

generation of an astaxanthin producing strain of *R. sphaeroides* using ΔREG(Δ5:YI), described above. See also Table 7 for further description of the strains and plasmids that were used in this example. Using the gene insertion method described by Higuchi, R., et al, Nucleic Acid Res. 16: 7351-7367, the *crtA* gene of ΔREG(Δ5:YI) was replaced by the *gps* gene from *A. fulgidus* to generate the strain ΔREG(Δ5:YI)(ΔA:gps). Electrocompetent cells ΔREG(Δ5:YI)(ΔA:gps) were generated as described above.

The construct pPgpsWZ was produced by cloning the *crtW* gene from *B. aurantiacum*, the *crtZ* gene from *P.stewartii*, and the *gps* gene from *A fulgidus* into the pPctrl plasmid using appropriate restriction enzymes. The construct pPWZ was produced by cloning the *crtW* gene from *B. aurantiacum* and the *crtZ* gene from *P.stewartii* into the pPctrl plasmid using appropriate restriction enzymes.

The pPWZ or pPgpsWZ constructs were electroporated into electrocompetent ΔREG(Δ5:YI)(ΔA:gps) as described earlier to generate ΔREG(Δ5:YI)(ΔA:gps)::pPWZ or ΔREG(Δ5:YI)(ΔA:gps)::pPgpsWZ, respectively. Transformation mixtures were plated out onto LB plates containing 50 μg/ml kanamycin. PCR analyses using PCR primers specific for *crtZ* were used to confirm the presence of the pPWZ or pPgpsWZ plasmids in ΔREG(Δ5:YI)(ΔA:gps).

Single colonies of ΔREG(Δ5:YI)(ΔA:gps)::pPWZ or ΔREG(Δ5:YI)(ΔA:gps)::pPgpsWZ were grown up in media supplemented with 50 μg/ml kanamycin as described earlier. Cell pellets were washed with distilled water and then carotenoids were extracted using acetone:methanol (7:2) at 30°C for 30 mins with shaking at 225 rpm. Carotenoid analysis was performed using LCMS analysis described above. The UV-Vis absorption spectra and the retention time using HPLC were used as diagnostic features for astaxanthin identification in ΔREG(Δ5:YI)(ΔA:gps)::pPWZ and ΔREG(Δ5:YI)(ΔA:gps)::pPgpsWZ, as well as the molecular ion and fragmentation patterns generated during mass spectrometry. The production of astaxanthin was confirmed in both ΔREG(Δ5:YI)(ΔA:gps)::pPWZ and ΔREG(Δ5:YI)(ΔA:gps)::pPgpsWZ. Increased astaxanthin production was observed in ΔREG(Δ5:YI)(ΔA:gps)::pPgpsWZ.

Example 7: Cloning and sequencing of a novel multifunctional Geranylgeranyl pyrophosphate synthase gene (*gps*) from *Sulfolobus shibatae*:

Degenerate primer sequences MFGGPP1 (5'CCAYGAYGAYATWATGGA3', SEQ ID NO:40) and MFGGPP2 (5'YTTYTTVCCYTYCCTAAT3', SEQ ID NO:41) were designed based on conserved sequences in *gps* gene sequences from *Sulfolobus solfotaricus* and *Sulfolobus acidocaldarius* and synthesized by Integrated DNA Technologies (Coralville, IA). PCR was performed in a mastercycler gradient machine (Eppendorf) with genomic DNA from *S. shibatae* (ATCC Accession No. 51178, lot # 1162977). Reaction conditions included five minutes at 96°C, followed by 30 cycles of denaturation at 94°C for 30 sec., annealing at 50 + 10°C for 60 sec., and extension at 72°C for 90 sec., and a final 72°C incubation for 10 min. An approximately 500-bp PCR product was obtained and cloned into the vector pC-BuntII-TOPO (Invitrogen Corp. Carlsbad, CA).

Independent clones were sequenced using the universal M13 forward and reverse primers. DNA sequencing was carried out at the AGAC, University of Minnesota, St. Paul, MN. DNA sequence analysis of this PCR product indicated similarity to the *gps* genes from *S. sulfotaricus* and *S. acidocaldarius*. The Universal Genome Walker kit (Clontech) was used to obtain more of the *gps* gene sequence flanking the original PCR product from *S. shibatae*. Primers were synthesized based on the partial sequence and used for genome walking experiments.

The following strategy was used to completely sequence the *S. shibatae gps* gene. The ERWCRTS homolog was observed upstream of the *S. sulfotaricus gps* gene. The UDP-A-acetylglucosamine—Dolichyl-phosphate-N-acetylglucosamine phosphotransferase gene was present downstream of the gps gene in both *S. sulfotaricus* and *S. acidocaldarius*. Primers were designed based on the sequence of the two genes SsDolidn (5'ACAGCGTTGGACACTCAG 3', SEQ ID NO:42) and SsERCRTup (5' GCGTCGATAATGGAAGTGAG 3', SEQ ID NO:43) of the *gps* gene. An approximately 2 kb PCR product was amplified using the SsDolidn and SsERCRTup primers and genomic DNA from *S. shibatae*. This PCR product was cloned into the vector pC-BuntII-TOPO as described above and sequenced using the universal M13 forward and reverse primers. The nucleotide sequence of the *gps* gene from *S. shibatae* is presented in SEQ

ID NO: 44, and the amino acid sequence of the protein encoded by the *gps* gene is presented in SEQ ID NO:45.

OTHER EMBODIMENTS

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

WHAT IS CLAIMED IS:

1. An isolated nucleic acid having at least 76% sequence identity to the nucleotide sequence of SEQ ID NO:1 or to a fragment of SEQ ID NO:1 at least 33 contiguous nucleotides in length.

2. The isolated nucleic acid of claim 1, said nucleic acid having at least 80% sequence identity to the nucleotide sequence of SEQ ID NO:1.

3. The isolated nucleic acid of claim 1, said nucleic acid having at least 85% sequence identity to the nucleotide sequence of SEQ ID NO:1.

4. The isolated nucleic acid of claim 1, said nucleic acid having at least 90% sequence identity to the nucleotide sequence of SEQ ID NO:1.

5. The isolated nucleic acid of claim 1, said nucleic acid having at least 95% sequence identity to the nucleotide sequence of SEQ ID NO:1.

6. An expression vector comprising the nucleic acid of claim 1 operably linked to an expression control element.

7. An isolated nucleic acid encoding a zeaxanthin glucosyl transferase polypeptide at least 75% identical to the amino acid sequence of SEQ ID NO:2.

8. An isolated nucleic acid having at least 78% sequence identity to the nucleotide sequence of SEQ ID NO:3 or to a fragment of SEQ ID NO:3 at least 32 contiguous nucleotides in length.

9. The isolated nucleic acid of claim 8, said nucleic acid having at least 80% sequence identity to the nucleotide sequence of SEQ ID NO:3.

10. The isolated nucleic acid of claim 8, said nucleic acid having at least 85% sequence identity to the nucleotide sequence of SEQ ID NO:3.

11. The isolated nucleic acid of claim 8, said nucleic acid having at least 90% sequence identity to the nucleotide sequence of SEQ ID NO:3.

12. The isolated nucleic acid of claim 8, said nucleic acid having at least 95% sequence identity to the nucleotide sequence of SEQ ID NO:3.

13. An expression vector comprising the nucleic acid of claim 8 operably linked to an expression control element.

14. An isolated nucleic acid encoding a lycopene β-cyclase polypeptide at least 83% identical to the amino acid sequence of SEQ ID NO:4.

15. An isolated nucleic acid having at least 81% sequence identity to the nucleotide sequence of SEQ ID NO:5 or to a fragment of SEQ ID NO:5 at least 60 contiguous nucleotides in length.

16. The isolated nucleic acid of claim 15, said nucleic acid having at least 85% sequence identity to the nucleotide sequence of SEQ ID NO:5.

17. The isolated nucleic acid of claim 15, said nucleic acid having at least 90% sequence identity to the nucleotide sequence of SEQ ID NO:5.

18. The isolated nucleic acid of claim 15, said nucleic acid having at least 95% sequence identity to the nucleotide sequence of SEQ ID NO:5.

19. An expression vector comprising the nucleic acid of claim 15 operably linked to an expression control element.

20. An isolated nucleic acid encoding a geranylgeranyl pyrophosphate synthase polypeptide at least 85% identical to the amino acid sequence of SEQ ID NO:6.

21. An isolated nucleic acid having at least 82% sequence identity to the nucleotide sequence of SEQ ID NO:7 or to a fragment of SEQ ID NO:7 at least 30 contiguous nucleotides in length.

22. The isolated nucleic acid of claim 21, said nucleic acid having at least 85% sequence identity to the nucleotide sequence of SEQ ID NO:7.

23. The isolated nucleic acid of claim 21, said nucleic acid having at least 90% sequence identity to the nucleotide sequence of SEQ ID NO:7.

24. The isolated nucleic acid of claim 21, said nucleic acid having at least 95% sequence identity to the nucleotide sequence of SEQ ID NO:7.

25. An expression vector comprising the nucleic acid of claim 21 operably linked to an expression control element.

26. An isolated nucleic acid encoding a phytoene desaturase polypeptide at least 90% identical to the amino acid sequence of SEQ ID NO:8.

27. An isolated nucleic acid having at least 82% sequence identity to the nucleotide sequence of SEQ ID NO:9 or to a fragment of SEQ ID NO:9 at least 23 contiguous nucleotides in length.

28. The isolated nucleic acid of claim 27, said nucleic acid having at least 85% sequence identity to the nucleotide sequence of SEQ ID NO:9.

29. The isolated nucleic acid of claim 27, said nucleic acid having at least 90% sequence identity to the nucleotide sequence of SEQ ID NO:9.

30. The isolated nucleic acid of claim 27, said nucleic acid having at least 95% sequence identity to the nucleotide sequence of SEQ ID NO:9.

31. An expression vector comprising the nucleic acid of claim 27 operably linked to an expression control element.

32. An isolated nucleic acid encoding a phytoene synthase polypeptide at least 89% identical to the amino acid sequence of SEQ ID NO:10.

33. An isolated nucleic acid having at least 85% sequence identity to the nucleotide sequence of SEQ ID NO:11 or to a fragment of SEQ ID NO:11 at least 36 contiguous nucleotides in length.

34. The isolated nucleic acid of claim 33, said nucleic acid having at least 85% sequence identity to the nucleotide sequence of SEQ ID NO:11.

35. The isolated nucleic acid of claim 33, said nucleic acid having at least 90% sequence identity to the nucleotide sequence of SEQ ID NO:11.

36. The isolated nucleic acid of claim 33, said nucleic acid having at least 95% sequence identity to the nucleotide sequence of SEQ ID NO:11.

37. An expression vector comprising the nucleic acid of claim 33 operably linked to an expression control element.

38. An isolated nucleic acid encoding a β-carotene hydroxylase polypeptide at least 90% identical to the amino acid sequence of SEQ ID NO:12.

39. Membranous bacteria comprising at least one exogenous nucleic acid encoding phytoene desaturase, lycopene β-cyclase, β-carotene hydroxylase, and β-carotene C4

oxygenase, wherein expression of said at least one exogenous nucleic acid produces detectable amounts of astaxanthin in said membranous bacteria.

40. The membranous bacteria of claim 39, wherein the amino acid sequence of said phytoene desaturase is at least 90% identical to the amino acid sequence of SEQ ID NO:8.

41. The membranous bacteria of claim 39, wherein the amino acid sequence of said lycopene β-cyclase is at least 83% identical to the amino acid sequence of SEQ ID NO:4.

42. The membranous bacteria of claim 39, wherein the amino acid sequence of said β-carotene hydroxylase is at least 90% identical to the amino acid sequence of SEQ ID NO:12.

43. The membranous bacteria of claim 39, wherein said membranous bacteria further comprises an exogenous nucleic acid encoding geranylgeranyl pyrophosphate synthase.

44. The membranous bacteria of claim 39, wherein said membranous bacteria lacks endogenous bacteriochlorophyll biosynthesis.

45. The membranous bacteria of claim 43, wherein said exogenous nucleic acid encodes a multifunctional geranylgeranyl pyrophosphate synthase.

46. The membranous bacteria of claim 45, wherein the amino acid sequence of said multifunctional geranylgeranyl pyrophosphate synthase is at least 90% identical to the amino acid sequence of SEQ ID NO:45.

47. The membranous bacteria of claim 39, wherein the amino acid sequence of said β-carotene C4 oxygenase is at least 80% identical to the amino acid sequence of SEQ ID NO:39.

48. The membranous bacteria of claim 39, wherein said membranous bacteria further comprise an exogenous nucleic acid encoding phytoene synthase.

49. The membranous bacteria of claim 48, wherein the amino acid sequence of said phytoene synthase is at least 89% identical to the amino acid sequence of SEQ ID NO:10.

50. The membranous bacteria of claim 39, wherein said membranous bacteria are a *Rhodobacter* species.

51. Membranous bacteria, said membranous bacteria comprising an exogenous nucleic acid encoding a phytoene desaturase having an amino acid sequence at least 90% identical to the amino acid sequence of SEQ ID NO:8, and wherein said membranous bacteria produces detectable amounts of lycopene.

52. The membranous bacteria of claim 51, wherein said membranous bacteria further comprise a lycopene β-cyclase, and wherein said membranous bacteria produce detectable amounts of β-carotene.

53. The membranous bacteria of claim 52, wherein said membranous bacteria further comprise a β-carotene hydroxylase, and wherein said membranous bacteria produce detectable amounts of zeaxanthin.

54. Membranous bacteria comprising at least one exogenous nucleic acid encoding phytoene desaturase, lycopene β-cyclase, and β-carotene C4 oxygenase, wherein expression of said at least one exogenous nucleic acid produces detectable amounts of canthaxanthin in said membranous bacteria.

55. A composition comprising an engineered *Rhodobacter* cell, wherein said cell produces a detectable amount of astaxanthin or canthaxanthin.

56. The composition of claim 55, wherein said engineered *Rhodobacter* cell comprises at least one exogenous nucleic acid encoding phytoene desaturase, lycopene β-cyclase, β-carotene hydroxylase, and β-carotene C4 oxygenase.

57. The composition of claim 55, wherein said composition is formulated for aquaculture.

58. The composition of claim 57, wherein said composition pigments the flesh of fish or the carapace of crustaceans after ingestion.

59. The composition of claim 55, wherein said composition is formulated for human consumption.

60. The composition of claim 55, wherein said composition is formulated as an animal feed.

61. The composition of claim 60, wherein said animal feed is formulated for consumption by chickens, turkeys, cattle, swine, or sheep.

62. A method of making a nutraceutical, said method comprising extracting carotenoids from an engineered *Rhodobacter* cell, said engineered *Rhodobacter* cell comprising at least one exogenous nucleic acid encoding phytoene desaturase, lycopene β-cyclase, β-carotene hydroxylase, and β-carotene C4 oxygenase, and wherein said *Rhodobacter* cell produces detectable amounts of astaxanthin.

63. Membranous bacteria, said membranous bacteria comprising an exogenous nucleic acid encoding a lycopene β-cyclase having an amino acid sequence at least 83% identical to the amino acid sequence of SEQ ID NO:4.

64. The membranous bacteria of claim 63, said membranous bacteria further comprising a phytoene desaturase, wherein said membranous bacteria produces detectable amounts of β-carotene.

65. The membranous bacteria of claim 64, said membranous bacteria further comprising a β-carotene hydroxylase, wherein said bacteria produces detectable amounts of zeaxanthin.

66. Membranous bacteria, said membranous bacteria comprising a β-carotene hydroxylase having an amino acid sequence at least 90% identical to the amino acid sequence of SEQ ID NO:12.

67. The membranous bacteria of claim 66, said membranous bacteria further comprising a lycopene β-cyclase, and wherein said membranous bacteria produces detectable amounts of zeaxanthin.

68. The membranous bacteria of claim 67, said membranous bacteria further comprising a phytoene desaturase, wherein said membranous bacteria produces detectable amounts of β-carotene.

69. Membranous bacteria, said bacteria lacking an endogenous nucleic acid encoding a farnesyl pyrophosphate synthase, and wherein said bacteria produce detectable amounts of carotenoids.

70. The membranous bacteria of claim 69, wherein said bacteria comprise an exogenous nucleic acid encoding a multifunctional geranylgeranyl pyrophosphate synthase.

71. The membranous bacteria of claim 70, wherein the amino acid sequence of said multifunctional geranylgeranyl pyrophosphate synthase is at least 90% identical to the amino acid sequence of SEQ ID NO:45.

72. The membranous bacteria of claim 69, wherein said membranous bacteria are a species of *Rhodobacter*.

73. An isolated nucleic acid having at least 60% sequence identity to the nucleotide sequences of SEQ ID NO:38, or to a fragment of the nucleic acid of SEQ ID NO:38 at least 15 contiguous nucleotides in length.

74. The isolated nucleic acid of claim 73, said nucleic acid having at least 80% sequence identity to the nucleotide sequences of SEQ ID NO:38, or to a fragment of the nucleic acid of SEQ ID NO:38 at least 15 contiguous nucleotides in length.

75. The isolated nucleic acid of claim 73, said nucleic acid having at least 90% sequence identity to the nucleotide sequences of SEQ ID NO:38, or to a fragment of the nucleic acid of SEQ ID NO:38 at least 15 contiguous nucleotides in length.

76. The isolated nucleic acid of claim 73, wherein said nucleic acid encodes a β-carotene C4 oxygenase.

77. Membranous bacteria comprising an exogenous nucleic acid encoding a β-carotene C4 oxygenase, said β-carotene oxygenase having an amino acid sequence at least 80% identical to the amino acid sequence of SEQ ID NO:39.

78. A host cell comprising an exogenous nucleic acid, wherein the exogenous nucleic acid comprises a nucleic acid sequence encoding one or more polypeptides that catalyze the formation of (3S, 3'S) astaxanthin, wherein the host cell produces CoQ-10 and (3S, 3'S) astaxanthin.

79. A method of making CoQ-10 and (3S, 3'S) astaxanthin at substantially the same time, the method comprising transforming a host cell with a nucleic acid, wherein the nucleic acid comprises a nucleic acid sequence that encodes one or more

polypeptides, wherein the polypeptides catalyze the formation of (3S, 3'S) astaxanthin; and culturing the host cell under conditions that allow for the production of (3S, 3'S) astaxanthin and CoQ-10.

80. The method of claim 79, additionally comprising transforming the host cell with at least one exogenous nucleic acid, the exogenous nucleic acid encoding one or more polypeptides, wherein the polypeptides catalyze the formation of CoQ-10.

81. An isolated nucleic acid having a nucleotide sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:38, and SEQ ID NO:44.

82. An isolated nucleic acid having at least 90% sequence identity to the nucleotide sequences of SEQ ID NO:44, or to a fragment of the nucleic acid of SEQ ID NO:44 at least 60 contiguous nucleotides in length.

83. A method of making geranylgeranyl pyrophosphate, said method comprising contacting isopentenyl pyrophosphate and dimethylallyl pyrophosphate with a polypeptide encoded by the isolated nucleic acid of claim 82.

84. A method of making geranylgeranyl pyrophosphate, said method comprising contacting farnesyl pyrophosphate and isopentenyl pyrophosphate with a polypeptide encoded by the isolated nucleic acid of claim 15 or the polypeptide of claim 20.

85. A method of making β-carotene, said method comprising contacting lycopene with a polypeptide encoded by the isolated nucleic acid of claim 8 or the polypeptide of claim 14.

86. A method of making lycopene, said method comprising contacting phytoene with a polypeptide encoded by the isolated nucleic acid of claim 21 or the polypeptide of claim 26.

87. A method of making phytoene, said method comprising contacting geranylgeranyl pyrophosphate with a polypeptide encoded by the isolated nucleic acid of claim 27 or the polypeptide of claim 32.

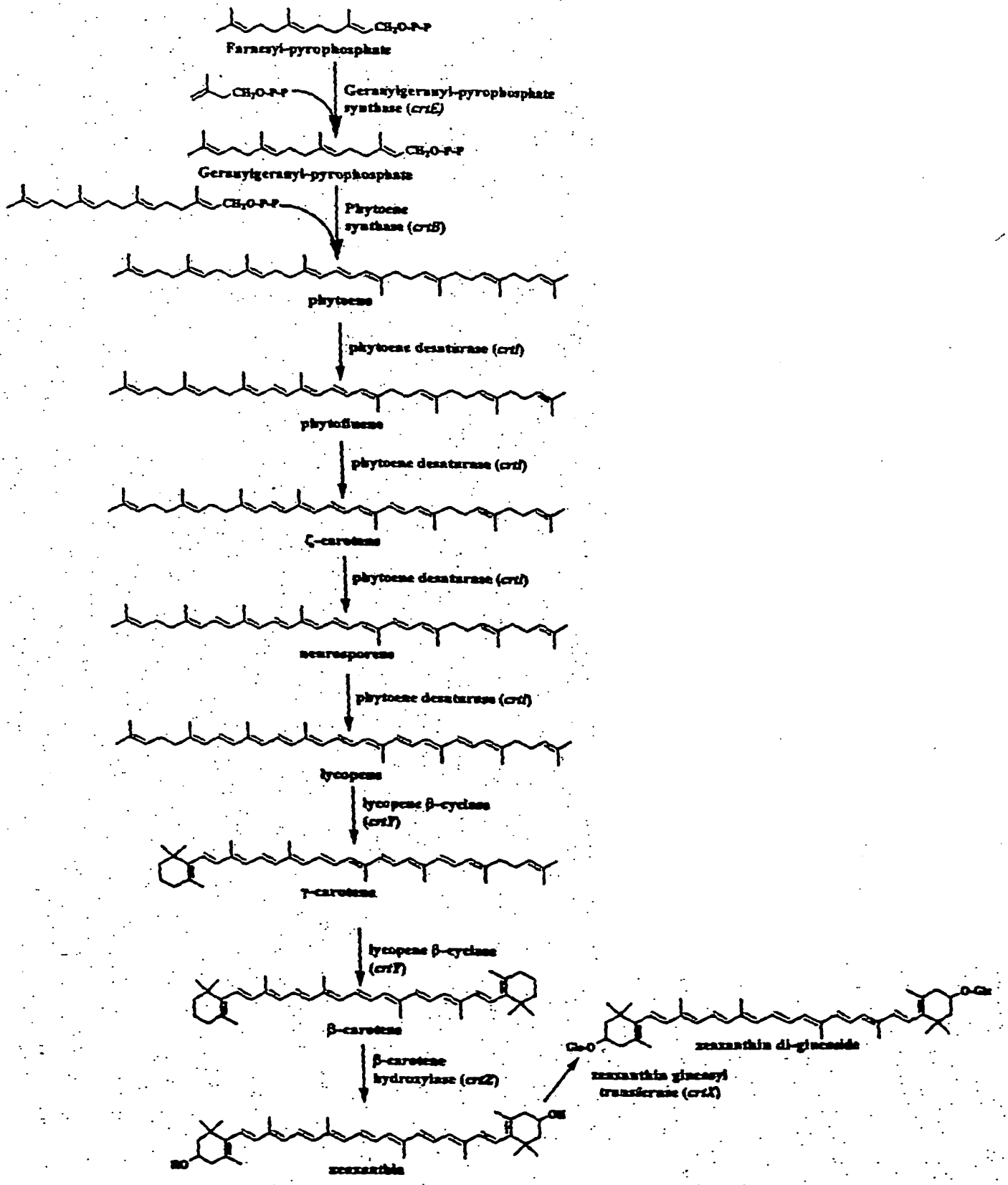
88. A method of making zeaxanthin, said method comprising contacting β-carotene with a polypeptide encoded by the isolated nucleic acid of claim 33 or the polypeptide of claim 38.

89. A method of making canthaxanthin, said method comprising contacting β-carotene with a polypeptide encoded by the isolated nucleic acid of claim 73 or a polypeptide having an amino acid sequence at least 80% identical to the amino acid sequence of SEQ ID NO:39.

90. A method of making astaxanthin, said method comprising contacting canthaxanthin with a polypeptide encoded by the isolated nucleic acid sequence of claim 33 or the polypeptide of claim 38.

91. A method of making astaxanthin, said method comprising contacting zeaxanthin with a polypeptide encoded by the isolated nucleic acid sequence of claim 73 or a polypeptide having an amino acid sequence at least 80% identical to the amino acid sequence of SEQ ID NO:39.

Figure 1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phytofluene
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ζ-carotene
phytoene desaturase (crtI)
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lycopene
lycopene β-cyclase (crtY)
γ-carotene
lycopene β-cyclase (crtY)
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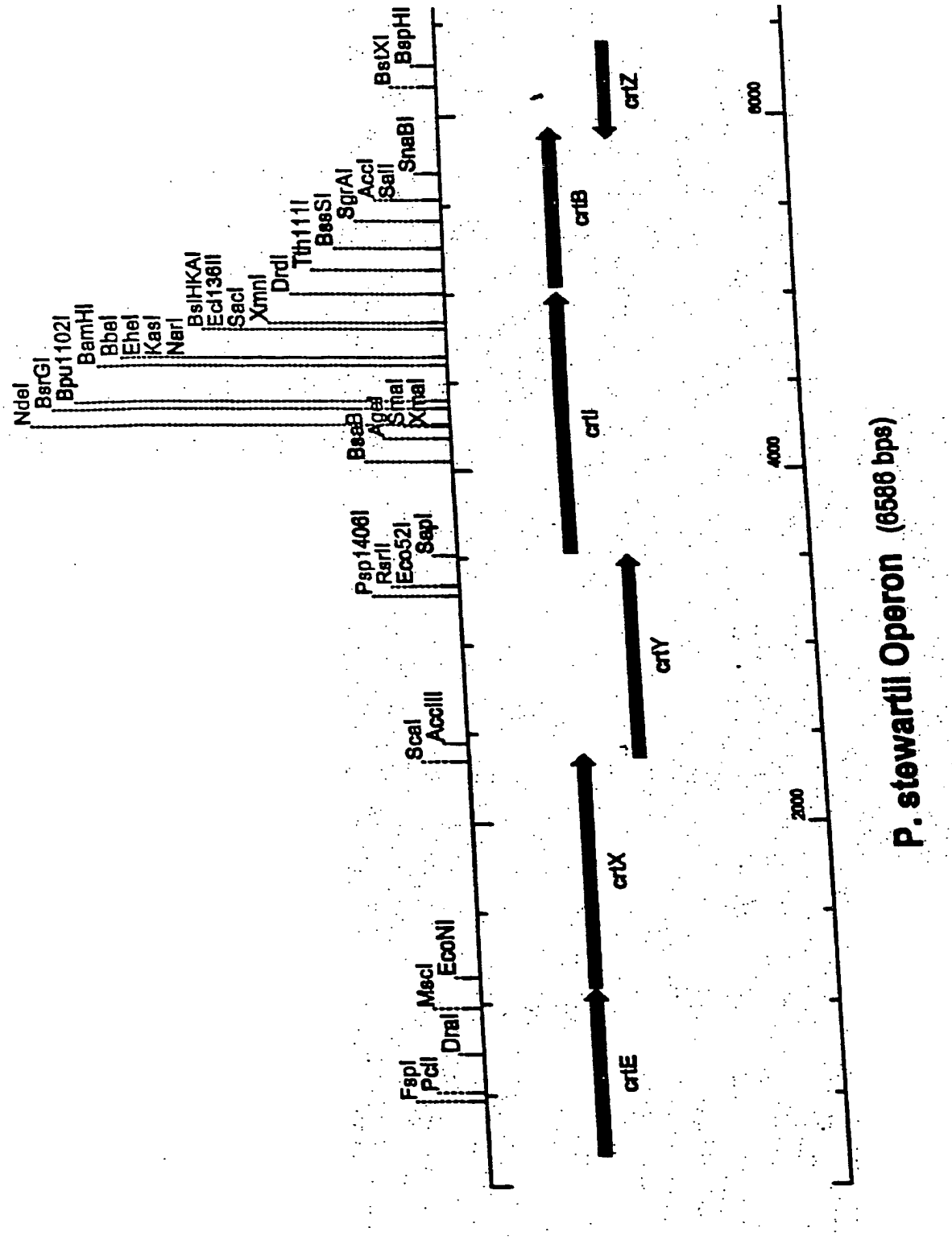
zeaxanthin di-glucoside

Figure 2



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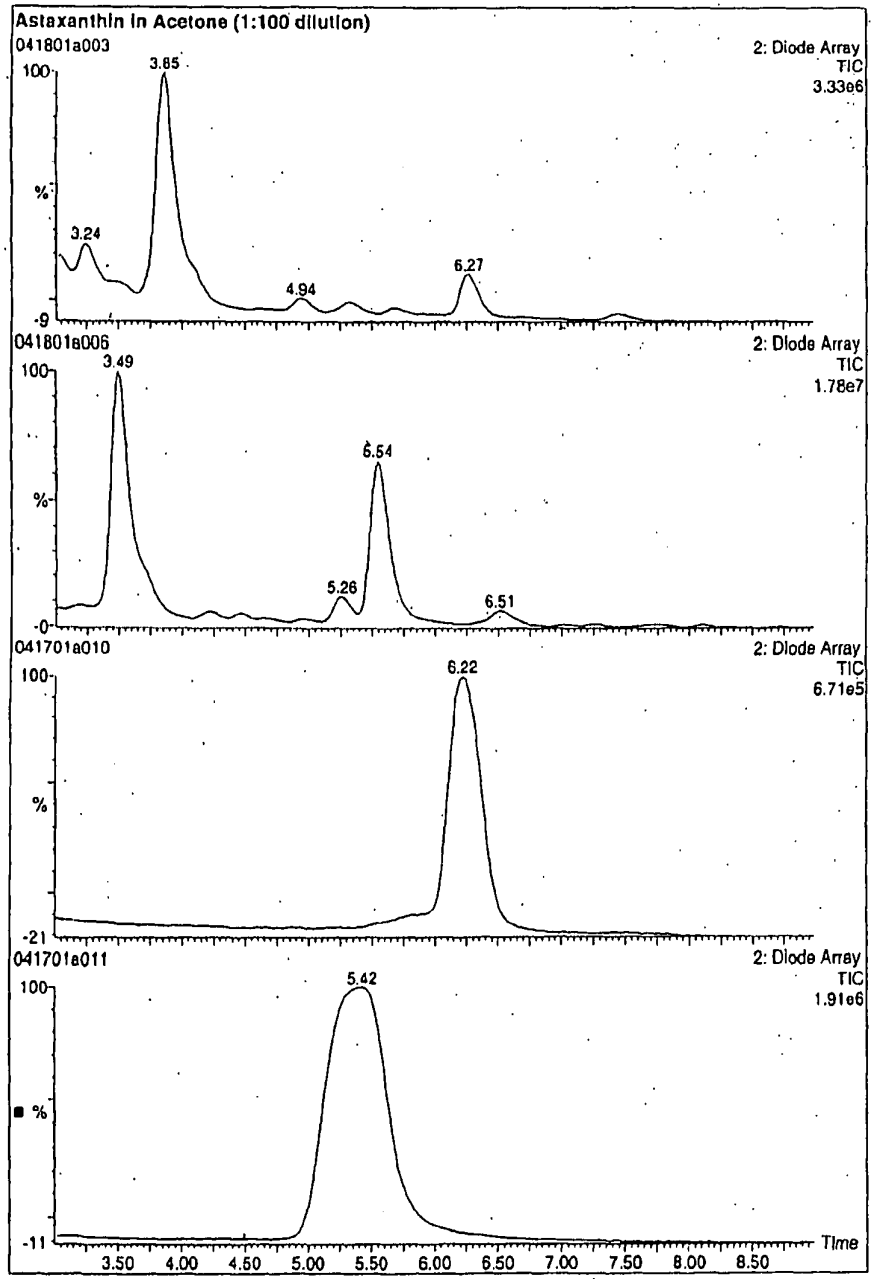

Pantoea stewartii – zeaxanthin production

P.stewartii:: crtW (*Brevundimonas aurantiaca*) – astaxanthin production

Zeaxanthin standard

Astaxanthin standard

SEQUENCE LISTING

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Val Val Asp Phe Ala Asp Gln Ser Ala Ala Leu Ser Gln Ala Gln Leu
305                 310                 315                 320
Thr Ile Thr His Gly Gly Met Asn Thr Val Leu Asp Ala Ile Ala Ser
                325                 330                 335
Arg Thr Pro Leu Leu Ala Leu Pro Leu Ala Phe Asp Gln Pro Gly Val
            340                 345                 350
Ala Ser Arg Ile Val Tyr His Gly Ile Gly Lys Arg Ala Ser Arg Phe
        355                 360                 365
Thr Thr Ser His Ala Leu Ala Arg Gln Ile Arg Ser Leu Leu Thr Asn
    370                 375                 380
Thr Asp Tyr Pro Gln Arg Met Thr Lys Ile Gln Ala Ala Leu Arg Leu
385                 390                 395                 400
Ala Gly Gly Thr Pro Ala Ala Ala Asp Ile Val Glu Gln Ala Met Arg
                405                 410                 415
Thr Cys Gln Pro Val Leu Ser Gly Gln Asp Tyr Ala Thr Ala Leu
            420                 425                 430
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<210> 3
<211> 1149
<212> DNA
<213> Pantoea stewartii

<400> 3
atgcaaccgc actatgatct cattctggtc ggtgccggtc tggctaatgg ccttatcgcg 60
ctccggcttc agcaacagca tccggatatg cggatcttgc ttattgaggc gggtcctgag 120
gcgggaggga accatacctg gtcctttcac gaagaggatt taacgctgaa tcagcatcgc 180
tggatagcgc cgcttgtggt ccatcactgg cccgactacc aggttcgttt cccccaacgc 240
cgtcgccatg tgaacagtgg ctactactgc gtgacctccc ggcatttcgc cgggatactc 300
cggcaacagt ttggacaaca tttatggctg cataccgcgg tttcagccgt tcatgctgaa 360
tcggtccagt tagcggatgg ccggattatt catgccagta cagtgatcga cggacggggt 420
tacacgcctg attctgcact acgcgtagga ttccaggcat ttatcggtca ggagtggcaa 480
ctgagcgcgc cgcatggttt atcgtcaccg attatcatgg atgcgacggt cgatcagcaa 540
aatggctacc gctttgttta taccctgccg ctttccgcaa ccgcactgct gatcgaagac 600
acacactaca ttgacaaggc taatcttcag gccgaacggg cgcgtcagaa cattcgcgat 660
tatgctgcgc gacagggttg gccgttacag acgttgctgc gggaagaaca gggtgcattg 720
cccattacgt taacgggcga taatcgtcag ttttggcaac agcaaccgca agcctgtagc 780
ggattacgcg ccgggctgtt tcatccgaca accggctact ccctaccgct cgcggtggcg 840
ctggccgatc gtctcagcgc gctggatgtg tttacctctt cctctgttca ccagacgatt 900
gctcactttg cccagcaacg ttggcagcaa caggggtttt tccgcatgct gaatcgcatg 960
ttgtttttag ccggaccggc cgagtcacgc tggcgtgtga tgcagcgttt ctatggctta 1020
cccgaggatt tgattgcccg cttttatgcg ggaaaactca ccgtgaccga tcggctacgc 1080
attctgagcg gcaagccgcc cgttcccgtt ttcgcggcat tgcaggcaat tatgacgact 1140
catcgttga 1149

<210> 4
<211> 382
<212> PRT
<213> Pantoea stewartii

<400> 4
Met Gln Pro His Tyr Asp Leu Ile Leu Val Gly Ala Gly Leu Ala Asn
1 5 10 15
Gly Leu Ile Ala Leu Arg Leu Gln Gln Gln His Pro Asp Met Arg Ile
20 25 30
Leu Leu Ile Glu Ala Gly Pro Glu Ala Gly Gly Asn His Thr Trp Ser
35 40 45
Phe His Glu Glu Asp Leu Thr Leu Asn Gln His Arg Trp Ile Ala Pro
50 55 60
Leu Val Val His His Trp Pro Asp Tyr Gln Val Arg Phe Pro Gln Arg
65 70 75 80
Arg Arg His Val Asn Ser Gly Tyr Tyr Cys Val Thr Ser Arg His Phe
85 90 95
Ala Gly Ile Leu Arg Gln Gln Phe Gly Gln His Leu Trp Leu His Thr
100 105 110
Ala Val Ser Ala Val His Ala Glu Ser Val Gln Leu Ala Asp Gly Arg
115 120 125
Ile Ile His Ala Ser Thr Val Ile Asp Gly Arg Gly Tyr Thr Pro Asp
130 135 140
Ser Ala Leu Arg Val Gly Phe Gln Ala Phe Ile Gly Gln Glu Trp Gln
145 150 155 160
Leu Ser Ala Pro His Gly Leu Ser Ser Pro Ile Ile Met Asp Ala Thr
165 170 175
Val Asp Gln Gln Asn Gly Tyr Arg Phe Val Tyr Thr Leu Pro Leu Ser
180 185 190
Ala Thr Ala Leu Leu Ile Glu Asp Thr His Tyr Ile Asp Lys Ala Asn
195 200 205
Leu Gln Ala Glu Arg Ala Arg Gln Asn Ile Arg Asp Tyr Ala Ala Arg
210 215 220

```
Gln Gly Trp Pro Leu Gln Thr Leu Leu Arg Glu Glu Gln Gly Ala Leu
225                 230                 235                 240
Pro Ile Thr Leu Thr Gly Asp Asn Arg Gln Phe Trp Gln Gln Gln Pro
                245                 250                 255
Gln Ala Cys Ser Gly Leu Arg Ala Gly Leu Phe His Pro Thr Thr Gly
            260                 265                 270
Tyr Ser Leu Pro Leu Ala Val Ala Leu Ala Asp Arg Leu Ser Ala Leu
        275                 280                 285
Asp Val Phe Thr Ser Ser Ser Val His Gln Thr Ile Ala His Phe Ala
    290                 295                 300
Gln Gln Arg Trp Gln Gln Gln Gly Phe Phe Arg Met Leu Asn Arg Met
305                 310                 315                 320
Leu Phe Leu Ala Gly Pro Ala Glu Ser Arg Trp Arg Val Met Gln Arg
                325                 330                 335
Phe Tyr Gly Leu Pro Glu Asp Leu Ile Ala Arg Phe Tyr Ala Gly Lys
            340                 345                 350
Leu Thr Val Thr Asp Arg Leu Arg Ile Leu Ser Gly Lys Pro Pro Val
        355                 360                 365
Pro Val Phe Ala Ala Leu Gln Ala Ile Met Thr Thr His Arg
    370                 375                 380
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<210> 5
<211> 912
<212> DNA
<213> Pantoea stewartii

<400> 5

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atgatggtct gcgcaaaaaa acacgttcac cttactggca tttcggctga gcagttgctg      60
gctgatatcg atagccgcct tgatcagtta ctgccggttc agggtgagcg ggattgtgtg     120
ggtgccgcga tgcgtgaagg cacgctggca ccgggcaaac gtattcgtcc gatgctgctg     180
ttattaacag cgcgcgatct tggctgtgcg atcagtcacg ggggattact ggatttagcc     240
tgcgcggttg aaatggtgca tgctgcctcg ctgattctgg atgatatgcc ctgcatggac     300
gatgcgcaga tgcgtcgggg gcgtcccacc attcacacgc agtacggtga acatgtggcg     360
attctggcgg cggtcgcttt actcagcaaa gcgtttgggg tgattgccga ggctgaaggt     420
ctgacgccga tagccaaaac tcgcgcggtg tcggagctgt ccactgcgat tggcatgcag     480
ggtctggttc agggccagtt taaggacctc tcggaaggcg ataaaccccg cagcgccgat     540
gccatactgc taaccaatca gtttaaaacc agcacgctgt tttgcgcgtc aacgcaaatg     600
gcgtccattg cggccaacgc gtcctgcgaa gcgcgtgaga acctgcatcg tttctcgctc     660
gatctcggcc aggcctttca gttgcttgac gatcttaccg atggcatgac cgataccggc     720
aaagacatca atcaggatgc aggtaaatca acgctggtca atttattagg ctcaggcgcg     780
gtcgaagaac gcctgcgaca gcatttgcgc ctggccagtg aacacctttc cgcggcatgc     840
caaaacggcc attccaccac ccaacttttt attcaggcct ggtttgacaa aaaactcgct     900
gccgtcagtt aa                                                        912
```

<210> 6
<211> 303
<212> PRT
<213> Pantoea stewartii

<400> 6

```
Met Met Val Cys Ala Lys Lys His Val His Leu Thr Gly Ile Ser Ala
 1               5                  10                  15
Glu Gln Leu Leu Ala Asp Ile Asp Ser Arg Leu Asp Gln Leu Leu Pro
            20                  25                  30
Val Gln Gly Glu Arg Asp Cys Val Gly Ala Ala Met Arg Glu Gly Thr
        35                  40                  45
Leu Ala Pro Gly Lys Arg Ile Arg Pro Met Leu Leu Leu Leu Thr Ala
```

```
    50                  55                  60
Arg Asp Leu Gly Cys Ala Ile Ser His Gly Gly Leu Leu Asp Leu Ala
65                  70                  75                  80
Cys Ala Val Glu Met Val His Ala Ala Ser Leu Ile Leu Asp Asp Met
                85                  90                  95
Pro Cys Met Asp Asp Ala Gln Met Arg Arg Gly Arg Pro Thr Ile His
            100                 105                 110
Thr Gln Tyr Gly Glu His Val Ala Ile Leu Ala Ala Val Ala Leu Leu
        115                 120                 125
Ser Lys Ala Phe Gly Val Ile Ala Glu Ala Glu Gly Leu Thr Pro Ile
    130                 135                 140
Ala Lys Thr Arg Ala Val Ser Glu Leu Ser Thr Ala Ile Gly Met Gln
145                 150                 155                 160
Gly Leu Val Gln Gly Gln Phe Lys Asp Leu Ser Glu Gly Asp Lys Pro
                165                 170                 175
Arg Ser Ala Asp Ala Ile Leu Leu Thr Asn Gln Phe Lys Thr Ser Thr
            180                 185                 190
Leu Phe Cys Ala Ser Thr Gln Met Ala Ser Ile Ala Ala Asn Ala Ser
        195                 200                 205
Cys Glu Ala Arg Glu Asn Leu His Arg Phe Ser Leu Asp Leu Gly Gln
    210                 215                 220
Ala Phe Gln Leu Leu Asp Asp Leu Thr Asp Gly Met Thr Asp Thr Gly
225                 230                 235                 240
Lys Asp Ile Asn Gln Asp Ala Gly Lys Ser Thr Leu Val Asn Leu Leu
                245                 250                 255
Gly Ser Gly Ala Val Glu Glu Arg Leu Arg Gln His Leu Arg Leu Ala
            260                 265                 270
Ser Glu His Leu Ser Ala Ala Cys Gln Asn Gly His Ser Thr Thr Gln
        275                 280                 285
Leu Phe Ile Gln Ala Trp Phe Asp Lys Lys Leu Ala Ala Val Ser
    290                 295                 300
```

<210> 7
<211> 1479
<212> DNA
<213> Pantoea stewartii

<400> 7

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atgaaaccaa ctacggtaat tggtgcgggc tttggtggcc tggcactggc aattcgttta      60
caggccgcag gtattcctgt tttgctgctt gagcagcgcg acaagccggg tggccgggct     120
tatgtttatc aggagcaggg ctttactttt gatgcaggcc ctaccgttat caccgatccc     180
agcgcgattg aagaactgtt tgctctggcc ggtaaacagc ttaaggatta cgtcgagctg     240
ttgccggtca cgccgtttta tcgcctgtgc tgggagtccg gcaaggtctt caattacgat     300
aacgaccagg cccagttaga agcgcagata cagcagttta atccgcgcga tgttgcgggt     360
tatcgagcgt tccttgacta ttcgcgtgcc gtattcaatg agggctatct gaagctcggc     420
actgtgcctt ttttatcgtt caaagacatg cttcgggccg cgccccagtt ggcaaagctg     480
caggcatggc gcagcgttta cagtaaagtt gccggctaca ttgaggatga gcatcttcgg     540
caggcgtttt cttttcactc gctcttagtg gggggaatc cgtttgcaac ctcgtccatt     600
tatacgctga ttcacgcgtt agaacgggaa tggggcgtct ggtttccacg cggtggaacc     660
ggtgcgctgg tcaatggcat gatcaagctg tttcaggatc tgggcggcga agtcgtgctt     720
aacgcccggg tcagtcatat ggaaaccgtt ggggacaaga ttcaggccgt gcagttggaa     780
gacggcagac ggtttgaaac ctgcgcggtg gcgtcgaacg ctgatgttgt acatacctat     840
cgcgatctgc tgtctcagca tcccgcagcc gctaagcagg cgaaaaaact gcaatccaag     900
cgtatgagta actcactgtt tgtactctat tttggtctca accatcatca cgatcaactc     960
gcccatcata ccgtctgttt tgggccacgc taccgtgaac tgattcacga aatttttaac    1020
catgatggtc tggctgagga tttttcgctt tatttacacg caccttgtgt cacggatccg    1080
tcactggcac cggaagggtg cggcagctat tatgtgctgg cgcctgttcc acacttaggc    1140
```

```
acggcgaacc tcgactgggc ggtagaagga ccccgactgc gcgatcgtat ttttgactac    1200
cttgagcaac attacatgcc tggcttgcga agccagttgg tgacgcaccg tatgtttacg    1260
ccgttcgatt tccgcgacga gctcaatgcc tggcaaggtt cggccttctc ggttgaacct    1320
attctgaccc agagcgcctg gttccgacca cataaccgcg ataagcacat tgataatctt    1380
tatctggttg gcgcaggcac ccatcctggc gcgggcattc ccggcgtaat cggctcggcg    1440
aaggcgacgg caggcttaat gctggaggac ctgatttga                           1479
```

<210> 8
<211> 492
<212> PRT
<213> Pantoea stewartii

<400> 8

```
Met Lys Pro Thr Thr Val Ile Gly Ala Gly Phe Gly Gly Leu Ala Leu
 1               5                  10                  15
Ala Ile Arg Leu Gln Ala Ala Gly Ile Pro Val Leu Leu Leu Glu Gln
            20                  25                  30
Arg Asp Lys Pro Gly Gly Arg Ala Tyr Val Tyr Gln Glu Gln Gly Phe
        35                  40                  45
Thr Phe Asp Ala Gly Pro Thr Val Ile Thr Asp Pro Ser Ala Ile Glu
    50                  55                  60
Glu Leu Phe Ala Leu Ala Gly Lys Gln Leu Lys Asp Tyr Val Glu Leu
65                  70                  75                  80
Leu Pro Val Thr Pro Phe Tyr Arg Leu Cys Trp Glu Ser Gly Lys Val
                85                  90                  95
Phe Asn Tyr Asp Asn Asp Gln Ala Gln Leu Glu Ala Gln Ile Gln Gln
            100                 105                 110
Phe Asn Pro Arg Asp Val Ala Gly Tyr Arg Ala Phe Leu Asp Tyr Ser
        115                 120                 125
Arg Ala Val Phe Asn Glu Gly Tyr Leu Lys Leu Gly Thr Val Pro Phe
    130                 135                 140
Leu Ser Phe Lys Asp Met Leu Arg Ala Ala Pro Gln Leu Ala Lys Leu
145                 150                 155                 160
Gln Ala Trp Arg Ser Val Tyr Ser Lys Val Ala Gly Tyr Ile Glu Asp
                165                 170                 175
Glu His Leu Arg Gln Ala Phe Ser Phe His Ser Leu Leu Val Gly Gly
            180                 185                 190
Asn Pro Phe Ala Thr Ser Ser Ile Tyr Thr Leu Ile His Ala Leu Glu
        195                 200                 205
Arg Glu Trp Gly Val Trp Phe Pro Arg Gly Gly Thr Gly Ala Leu Val
    210                 215                 220
Asn Gly Met Ile Lys Leu Phe Gln Asp Leu Gly Gly Glu Val Val Leu
225                 230                 235                 240
Asn Ala Arg Val Ser His Met Glu Thr Val Gly Asp Lys Ile Gln Ala
                245                 250                 255
Val Gln Leu Glu Asp Gly Arg Arg Phe Glu Thr Cys Ala Val Ala Ser
            260                 265                 270
Asn Ala Asp Val Val His Thr Tyr Arg Asp Leu Leu Ser Gln His Pro
        275                 280                 285
Ala Ala Ala Lys Gln Ala Lys Lys Leu Gln Ser Lys Arg Met Ser Asn
    290                 295                 300
Ser Leu Phe Val Leu Tyr Phe Gly Leu Asn His His His Asp Gln Leu
305                 310                 315                 320
Ala His His Thr Val Cys Phe Gly Pro Arg Tyr Arg Glu Leu Ile His
                325                 330                 335
Glu Ile Phe Asn His Asp Gly Leu Ala Glu Asp Phe Ser Leu Tyr Leu
            340                 345                 350
```

```
His Ala Pro Cys Val Thr Asp Pro Ser Leu Ala Pro Glu Gly Cys Gly
        355                 360                 365
Ser Tyr Tyr Val Leu Ala Pro Val Pro His Leu Gly Thr Ala Asn Leu
    370                 375                 380
Asp Trp Ala Val Glu Gly Pro Arg Leu Arg Asp Arg Ile Phe Asp Tyr
385                 390                 395                 400
Leu Glu Gln His Tyr Met Pro Gly Leu Arg Ser Gln Leu Val Thr His
                405                 410                 415
Arg Met Phe Thr Pro Phe Asp Phe Arg Asp Glu Leu Asn Ala Trp Gln
            420                 425                 430
Gly Ser Ala Phe Ser Val Glu Pro Ile Leu Thr Gln Ser Ala Trp Phe
        435                 440                 445
Arg Pro His Asn Arg Asp Lys His Ile Asp Asn Leu Tyr Leu Val Gly
    450                 455                 460
Ala Gly Thr His Pro Gly Ala Gly Ile Pro Gly Val Ile Gly Ser Ala
465                 470                 475                 480
Lys Ala Thr Ala Gly Leu Met Leu Glu Asp Leu Ile
                485                 490
```

<210> 9
<211> 893
<212> DNA
<213> Pantoea stewartii

<400> 9

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ccatggcggt tggctcgaaa agctttgcga ctgcatcgac gcttttcgac gccaaaaccc     60
gtcgcagcgt gctgatgctt tacgcatggt gccgccactg cgacgacgtc attgacgatc    120
aaacactggg ctttcatgcc gaccagccct cttcgcagat gcctgagcag cgcctgcagc    180
agcttgaaat gaaaacgcgt caggcctacg ccggttcgca aatgcacgag cccgcttttg    240
ccgcgtttca ggaggtcgcg atggcgcatg atatcgctcc cgcctacgcg ttcgaccatc    300
tggaaggttt tgccatggat gtgcgcgaaa cgcgctacct gacactggac gatacgctgc    360
gttattgcta tcacgtcgcc ggtgttgtgg gcctgatgat ggcgcaaatt atgggcgttc    420
gcgataacgc cacgctcgat cgcgcctgcg atctcgggct ggctttccag ttgaccaaca    480
ttgcgcgtga tattgtcgac gatgctcagg tgggccgctg ttatctgcct gaaagctggc    540
tggaagagga aggactgacg aaagcgaatt atgctgcgcc agaaaaccgg caggccttaa    600
gccgtatcgc cgggcgactg gtacgggaag cggaacccta ttacgtatca tcaatggccg    660
gtctggcaca attaccctta cgctcggcct gggccatcgc gacagcgaag caggtgtacc    720
gtaaaattgg cgtgaaagtt gaacaggccg gtaagcaggc ctgggatcat cgccagtcca    780
cgtccaccgc cgaaaaatta acgcttttgc tgacggcatc cggtcaggca gttacttccc    840
ggatgaagac gtatccaccc cgtcctgctc atctctggca gcgcccgatc tag           893
```

<210> 10
<211> 296
<212> PRT
<213> Pantoea stewartii

<400> 10

```
Met Ala Val Gly Ser Lys Ser Phe Ala Thr Ala Ser Thr Leu Phe Asp
 1               5                  10                  15
Ala Lys Thr Arg Arg Ser Val Leu Met Leu Tyr Ala Trp Cys Arg His
            20                  25                  30
Cys Asp Asp Val Ile Asp Asp Gln Thr Leu Gly Phe His Ala Asp Gln
        35                  40                  45
Pro Ser Ser Gln Met Pro Glu Gln Arg Leu Gln Gln Leu Glu Met Lys
    50                  55                  60
Thr Arg Gln Ala Tyr Ala Gly Ser Gln Met His Glu Pro Ala Phe Ala
65                  70                  75                  80
```

```
Ala Phe Gln Glu Val Ala Met Ala His Asp Ile Ala Pro Ala Tyr Ala
                85                  90                  95
Phe Asp His Leu Glu Gly Phe Ala Met Asp Val Arg Glu Thr Arg Tyr
            100                 105                 110
Leu Thr Leu Asp Asp Thr Leu Arg Tyr Cys Tyr His Val Ala Gly Val
        115                 120                 125
Val Gly Leu Met Met Ala Gln Ile Met Gly Val Arg Asp Asn Ala Thr
    130                 135                 140
Leu Asp Arg Ala Cys Asp Leu Gly Leu Ala Phe Gln Leu Thr Asn Ile
145                 150                 155                 160
Ala Arg Asp Ile Val Asp Asp Ala Gln Val Gly Arg Cys Tyr Leu Pro
                165                 170                 175
Glu Ser Trp Leu Glu Glu Glu Gly Leu Thr Lys Ala Asn Tyr Ala Ala
            180                 185                 190
Pro Glu Asn Arg Gln Ala Leu Ser Arg Ile Ala Gly Arg Leu Val Arg
        195                 200                 205
Glu Ala Glu Pro Tyr Tyr Val Ser Ser Met Ala Gly Leu Ala Gln Leu
    210                 215                 220
Pro Leu Arg Ser Ala Trp Ala Ile Ala Thr Ala Lys Gln Val Tyr Arg
225                 230                 235                 240
Lys Ile Gly Val Lys Val Glu Gln Ala Gly Lys Gln Ala Trp Asp His
                245                 250                 255
Arg Gln Ser Thr Ser Thr Ala Glu Lys Leu Thr Leu Leu Leu Thr Ala
            260                 265                 270
Ser Gly Gln Ala Val Thr Ser Arg Met Lys Thr Tyr Pro Pro Arg Pro
        275                 280                 285
Ala His Leu Trp Gln Arg Pro Ile
    290                 295
```

<210> 11
<211> 528
<212> DNA
<213> Pantoea stewartii

<400> 11

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atgttgtgga tttggaatgc cctgatcgtg tttgtcaccg tggtcggcat ggaagtggtt   60
gctgcactgg cacataaata catcatgcac ggctggggtt ggggctggca tctttcacat  120
catgaaccgc gtaaaggcgc atttgaagtt aacgatctct atgccgtggt attcgccatt  180
gtgtcgattg ccctgattta cttcggcagt acaggaatct ggccgctcca gtggattggt  240
gcaggcatga ccgcttatgg tttactgtat tttatggtcc acgacggact ggtacaccag  300
cgctggccgt tccgctacat accgcgcaaa ggctacctga aacggttata catggcccac  360
cgtatgcatc atgctgtaag gggaaaagag ggctgcgtgt cctttggttt tctgtacgcg  420
ccaccgttat ctaaacttca ggcgacgctg agagaaaggc atgcggctag atcgggcgct  480
gccagagatg agcaggacgg ggtggatacg tcttcatccg ggaagtaa               528
```

<210> 12
<211> 175
<212> PRT
<213> Pantoea stewartii

<400> 12

```
Met Leu Trp Ile Trp Asn Ala Leu Ile Val Phe Val Thr Val Val Gly
 1               5                  10                  15
Met Glu Val Val Ala Ala Leu Ala His Lys Tyr Ile Met His Gly Trp
            20                  25                  30
Gly Trp Gly Trp His Leu Ser His His Glu Pro Arg Lys Gly Ala Phe
        35                  40                  45
```

```
Glu Val Asn Asp Leu Tyr Ala Val Val Phe Ala Ile Val Ser Ile Ala
    50                  55                  60
Leu Ile Tyr Phe Gly Ser Thr Gly Ile Trp Pro Leu Gln Trp Ile Gly
65                  70                  75                  80
Ala Gly Met Thr Ala Tyr Gly Leu Leu Tyr Phe Met Val His Asp Gly
                85                  90                  95
Leu Val His Gln Arg Trp Pro Phe Arg Tyr Ile Pro Arg Lys Gly Tyr
            100                 105                 110
Leu Lys Arg Leu Tyr Met Ala His Arg Met His His Ala Val Arg Gly
        115                 120                 125
Lys Glu Gly Cys Val Ser Phe Gly Phe Leu Tyr Ala Pro Pro Leu Ser
    130                 135                 140
Lys Leu Gln Ala Thr Leu Arg Glu Arg His Ala Ala Arg Ser Gly Ala
145                 150                 155                 160
Ala Arg Asp Glu Gln Asp Gly Val Asp Thr Ser Ser Ser Gly Lys
                165                 170                 175
```

<210> 13
<211> 29
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer

<400> 13
atyatgcacg gctggggwtg gsgmtggca 29

<210> 14
<211> 31
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer

<400> 14
ggccarcgyt gatgcaccag mccgtcrtgc a 31

<210> 15
<211> 26
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer

<400> 15
ctgatgctct aygcctggtg ccgcca 26

<210> 16
<211> 23
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer

<400> 16
tcgcgrgcra trttsgtcar ctg 23

<210> 17
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer

<400> 17
atbmtsatgg aygcsacsgt 20

<210> 18
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer

<400> 18
ytratcgarg ayacgcrcta 20

<210> 19
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer

<400> 19
rsggcagyga atagccrgtg 20

<210> 20
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer

<400> 20
aacagcatsc grttcagcak gcgsa 25

<210> 21
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer

<400> 21

ccgacggtka tcaccgatcc 20

<210> 22
<211> 19
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer

<400> 22
ctgcgccsac caggtagag 19

<210> 23
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer

<400> 23
ctygacgaya tgccctgcat ggac 24

<210> 24
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer

<400> 24
gtcgatttwc csgcgtcctk attg 24

<210> 25
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer

<400> 25
ggccgaattc caacgatgct ctggcagtta 30

<210> 26
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer

<400> 26
ggccagatct acttcaggcg acgctgagag 30

<210> 27
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer

<400> 27
ggccagatct tacgcgcggg taaagccaat 30

<210> 28
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer

<400> 28
ggcctctaga attaccgcgt ggttctgaag 30

<210> 29
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer

<400> 29
ggcctctaga tctgtacgcg ccaccgttat 30

<210> 30
<211> 27
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer

<400> 30
catcggtaag atcgtcaagc aactgaa 27

<210> 31
<211> 27
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer

<400> 31
gatttacctg catcctgatt gatgtct 27

<210> 32
<211> 27

<212> DNA
<213> Artificial Sequence

<220>
<223> Primer

<400> 32
atgtataacc gtttcaggta gcctttg 27

<210> 33
<211> 27
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer

<400> 33
aatacagtaa accataagcg gtcatgc 27

<210> 34
<211> 18
<212> DNA
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Phe Ile Val Ala His Asp Ala Met Tyr Gly Ser Leu Ala Pro Gly Arg
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Pro Arg Leu Asn Ala Ala Val Gly Arg Leu Thr Leu Gly Leu Tyr Ala
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Gly Phe Arg Phe Asp Arg Leu Lys Thr Ala His His Ala His His Ala
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Arg His Thr Asp Gln Pro Phe Ala Asp Ala His His Ala Arg Ser Ser
195 200 205
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35 40 45
Leu Ile Leu Val Ser Ser Ser Asp Leu Ile Gly Gly Asp Arg Gln Arg
50 55 60
Ala Tyr Lys Ala Ala Ala Ala Val Glu Ile Leu His Asn Phe Thr Leu
65 70 75 80
Val His Asp Asp Ile Met Asp Arg Asp Tyr Leu Arg Arg Gly Leu Pro
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Thr Val His Val Lys Trp Gly Glu Pro Met Ala Ile Leu Ala Gly Asp
100 105 110
Tyr Leu His Ala Lys Ala Phe Glu Ala Leu Asn Glu Ala Leu Lys Gly
115 120 125
Leu Asp Gly Asn Thr Phe Tyr Lys Ala Phe Ser Val Phe Ile Asn Ser
130 135 140
Ile Glu Ile Ile Ser Glu Gly Gln Ala Met Asp Met Ser Phe Glu Asn
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Arg Val Asp Val Thr Glu Glu Glu Tyr Met Gln Met Ile Lys Gly Lys
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Thr Ala Met Leu Phe Ser Cys Ser Ala Ala Leu Gly Gly Ile Ile Asn

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225                 230                 235                 240
Lys Lys Thr Ile Leu Val Ile Lys Thr Leu Ser Glu Ala Thr Asp Asp
                245                 250                 255
Glu Lys Lys Ile Leu Val Ser Thr Leu Gly Asn Arg Glu Ala Lys Lys
            260                 265                 270
Asp Asp Leu Glu Arg Ala Ser Glu Ile Ile Arg Lys Tyr Ser Leu Gln
        275                 280                 285
Tyr Ala Tyr Asn Leu Ala Lys Lys Tyr Ser Asp Leu Ala Leu Glu His
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(71) Applicant *(for all designated States except US)*: **CARGILL, INCORPORATED** [US/US]; P.O. Box 5624, Minneapolis, MN 55440-5624 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **DE SOUZA, Mervyn, L.** [US/US]; 10935 38th Avenue North, Plymouth, MN 55441 (US). **KOLLMANN, Sherry, R.** [US/US]; 12031 99th Avenue North, Maple Grove, MN 55369 (US). **MAY, Colleen, A.** [US/US]; 20 Gideons Point Road, Tonka Bay, MN 55331 (US). **SCHROEDER, William, A.** [US/US]; 3509 Highlands Road, Brooklyn Park, MN 55443 (US).

(74) Agent: **DEGRANDIS, Paula**; Cargill, Incorporated, P.O. Box 5624, Minneapolis, MN 55440-5624 (US).

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(54) Title: CAROTENOID BIOSYNTHESIS

(57) Abstract: Membranous bacteria that produce astaxanthin and other carotenoids are described, as well as isolated nucleic acids and expression vectors that can be used for producing carotenoids in microorganisms.

Carotenoid Biosynthesis

TECHNICAL FIELD

The invention relates to methods and materials for producing carotenoids, and in particular, to nucleic acid molecules, polypeptides, host cells, and methods that can be used for producing carotenoids.

BACKGROUND

Astaxanthin (3,3'-dihydroxy-β,β-carotene-4,4'-dione) is the primary carotenoid that imparts the pink pigment to the eggs, flesh, and skin of salmon, trout, and shrimp. Most animals cannot synthesize carotenoids. Rather, the pigments are acquired through the food chain from marine algae and phytoplankton, the primary producers of astaxanthin. ATX exists in three configurational isomers [(3S, 3'S), (3R, 3'R) and (3S, 3'R; 3R, 3'S)], however, ATX is found in the marine environment only in the (3S, 3'S) form. Consequently, this form is considered the natural and most desirable form of ATX.

Although astaxanthin has been commercially extracted from some yeast and crustacea species and has been chemically synthesized as a 1:2:1 mixture of the (3S,3'S)-, (3S,3'R)- and (3R,3'R)-isomers, astaxanthin is limited in availability and is expensive to purchase. See, Torrisen et al. (1989) Crit. Rev. Aquatic Sci. 1:209; and Mayer (1994) Pure Appl. Chem., 66:931-938. Thus, there is a need for a less expensive source of the naturally-occurring (3S,3'S) astaxanthin.

SUMMARY

The invention is based on methods and materials for producing carotenoids such as lycopene, zeaxanthin, zeaxanthin diglucoside, canthaxanthin, β-carotene, lutein, and astaxanthin. Such carotenoids can be used as nutritional supplements in humans and can be formulated for use in aquaculture or as an animal feed. The invention provides nucleic acid molecules that can be used to engineer host cells having the ability to produce particular carotenoids and polypeptides that can be used in cell-free systems to make particular carotenoids. The engineered cells described herein can be used to produce large quantities of carotenoids.

In one aspect, the invention features an isolated nucleic acid having at least 76% sequence identity to the nucleotide sequence of SEQ ID NO:1 (e.g., at least 80%, 85%, 90%, or 95% sequence identity to the nucleotide sequence of SEQ ID NO:1) or to a fragment of SEQ ID NO:1 at least 33 contiguous nucleotides in length. An isolated nucleic acid can encode a zeaxanthin glucosyl transferase polypeptide at least 75% identical to the amino acid sequence of SEQ ID NO:2. Expression vectors containing such nucleic acids operably linked to an expression control element also are featured.

In another aspect, the invention features an isolated nucleic acid having at least 78% sequence identity to the nucleotide sequence of SEQ ID NO:3 (e.g., at least 80%, 85%, 90%, or 95% sequence identity to the nucleotide sequence of SEQ ID NO:3) or to a fragment of SEQ ID NO:3 at least 32 contiguous nucleotides in length. An isolated nucleic acid can encode a lycopene β-cyclase polypeptide at least 83% identical to the amino acid sequence of SEQ ID NO:4. β-carotene can be made by contacting lycopene with a polypeptide encoded by such isolated nucleic acids. The invention also features an expression vector that includes such nucleic acids operably linked to an expression control element.

In yet another aspect, the invention features an isolated nucleic acid having at least 81% sequence identity to the nucleotide sequence of SEQ ID NO:5 (e.g., at least 85%, 90%, or 95% sequence identity to the nucleotide sequence of SEQ ID NO:5) or to a fragment of SEQ ID NO:5 at least 60 contiguous nucleotides in length. An isolated nucleic acid also can encode a geranylgeranyl pyrophosphate synthase polypeptide at least 85% identical to the amino acid sequence of SEQ ID NO:6. Geranylgeranyl pyrophosphate can be made by contacting farnesyl pyrophosphate and isopentenyl pyrophosphate with a polypeptide encoded by such nucleic acids. Expression vectors that include such nucleic acids operably linked to an expression control element also are featured.

Isolated nucleic acids having at least 82% sequence identity to the nucleotide sequence of SEQ ID NO:7 (e.g., at least 85%, 90%, or 95% sequence identity to the nucleotide sequence of SEQ ID NO:7) or to a fragment of SEQ ID NO:7 at least 30 contiguous nucleotides in length also are featured. An isolated nucleic acid also can encode a phytoene desaturase polypeptide at least 90% identical to the amino acid

sequence of SEQ ID NO:8. Lycopene can be made by contacting phytoene with a polypeptide encoded by such nucleic acids. An expression vector that includes such nucleic acids operably linked to an expression control element also is featured.

The invention also features an isolated nucleic acid having at least 82% sequence identity to the nucleotide sequence of SEQ ID NO:9 (e.g., at least 85%, 90%, or 95% sequence identity to the nucleotide sequence of SEQ ID NO:9) or to a fragment of SEQ ID NO:9 at least 23 contiguous nucleotides in length. An isolated nucleic acid also can encode a phytoene synthase polypeptide at least 89% identical to the amino acid sequence of SEQ ID NO:10. Phytoene can be made by contacting geranylgeranyl pyrophosphate with a polypeptide encoded by such nucleic acids. An expression vector that includes such nucleic acids operably linked to an expression control element also is featured.

In yet another aspect, the invention features an isolated nucleic acid having at least 85% sequence identity to the nucleotide sequence of SEQ ID NO:11 (e.g., at least 90% or 95% identity to the nucleotide sequence of SEQ ID NO:11) or to a fragment of SEQ ID NO:11 at least 36 contiguous nucleotides in length. An isolated nucleic acid can encode a β-carotene hydroxylase polypeptide at least 90% identical to the amino acid sequence of SEQ ID NO:12. Zeaxanthin can be made by contacting β-carotene with a polypeptide encoded by such nucleic acids. Astaxanthin can be made by contacting canthaxanthin with a polypeptide encoded by such nucleic acids. The invention also features an expression vector that includes such nucleic acids operably linked to an expression control element.

The invention also features membranous bacteria (e.g., a *Rhodobacter* species) that include at least one exogenous nucleic acid encoding phytoene desaturase, lycopene β-cyclase, β-carotene hydroxylase, and β-carotene C4 oxygenase, wherein expression of the at least one exogenous nucleic acid produces detectable amounts of astaxanthin in the membranous bacteria. The amino acid sequence of the phytoene desaturase can be at least 90% identical to the amino acid sequence of SEQ ID NO:8. The amino acid sequence of the lycopene β-cyclase can be at least 83% identical to the amino acid sequence of SEQ ID NO:4. The amino acid sequence of the β-carotene hydroxylase can be at least 90% identical to the amino acid sequence of SEQ ID NO:12. The amino acid sequence of the β-carotene C4 oxygenase can be at least 80% identical to the amino acid

sequence of SEQ ID NO:39. The membranous bacteria further can include an exogenous nucleic acid encoding geranylgeranyl pyrophosphate synthase (e.g., a multifunctional geranylgeranyl pyrophosphate synthase) or can lack endogenous bacteriochlorophyll biosynthesis. The multifunctional geranylgeranyl pyrophosphate synthase can have an amino acid sequence at least 90% identical to the amino acid sequence of SEQ ID NO:45. The membranous bacteria further can include an exogenous nucleic acid encoding phytoene synthase. The phytoene synthase can have an amino acid sequence at least 89% identical to the amino acid sequence of SEQ ID NO:10.

In another aspect, the invention features membranous bacteria that include an exogenous nucleic acid encoding a phytoene desaturase having an amino acid sequence at least 90% identical to the amino acid sequence of SEQ ID NO:8, and wherein the membranous bacteria produces detectable amounts of lycopene. The membranous bacteria further can include a lycopene β-cyclase, wherein the membranous bacteria produce detectable amounts of β-carotene. The membranous bacteria also can include a β-carotene hydroxylase, wherein the membranous bacteria produce detectable amounts of zeaxanthin.

In still yet another aspect, the invention feature membranous bacteria that include at least one exogenous nucleic acid encoding phytoene desaturase, lycopene β-cyclase, and β-carotene C4 oxygenase, wherein expression of the at least one exogenous nucleic acid produces detectable amounts of canthaxanthin in the membranous bacteria. The membranous bacteria also can include a β-carotene hydroxylase, wherein the membranous bacteria produce detectable amounts of astaxanthin.

The invention also features a composition that includes an engineered *Rhodobacter* cell, wherein the cell produces a detectable amount of astaxanthin or canthaxanthin. The engineered *Rhodobacter* cell can include at least one exogenous nucleic acid encoding phytoene desaturase, lycopene β-cyclase, β-carotene hydroxylase, and β-carotene C4 oxygenase. The composition can be formulated for aquaculture and can pigment the flesh of fish or the carapace of crustaceans after ingestion. The composition can be formulated for human consumption or as an animal feed (e.g., formulated for consumption by chickens, turkeys, cattle, swine, or sheep).

The invention also features a method of making a nutraceutical. The method includes extracting carotenoids from an engineered *Rhodobacter* cell, the engineered *Rhodobacter* cell including at least one exogenous nucleic acid encoding phytoene desaturase, lycopene β-cyclase, β-carotene hydroxylase, and β-carotene C4 oxygenase, and wherein the *Rhodobacter* cell produces detectable amounts of astaxanthin.

In yet another aspect, the invention features membranous bacteria, wherein the membranous bacteria include an exogenous nucleic acid encoding a lycopene β-cyclase having an amino acid sequence at least 83% identical to the amino acid sequence of SEQ ID NO:4. The membranous bacteria further can include a phytoene desaturase, (e.g., an exogenous phytoene desaturase), wherein the membranous bacteria produce detectable amounts of β-carotene. The membranous bacteria also can include a β-carotene hydroxylase (e.g., an exogenous β-carotene hydroxylase), wherein the bacteria produce detectable amounts of zeaxanthin.

Membranous bacteria that include a β-carotene hydroxylase having an amino acid sequence at least 90% identical to the amino acid sequence of SEQ ID NO:12 also is featured. The membranous bacteria further can include a lycopene β-cyclase (e.g., an exogenous lycopene β-cyclase), wherein the membranous bacteria produce detectable amounts of zeaxanthin. The membranous bacteria also can include a phytoene desaturase (e.g., an exogenous phytoene desaturase), wherein the membranous bacteria produce detectable amounts of β-carotene.

The invention also features membranous bacteria (e.g., a *Rhodobacter* species) lacking an endogenous nucleic acid encoding a farnesyl pyrophosphate synthase, wherein the bacteria produces detectable amounts of carotenoids. The membranous bacteria also can include an exogenous nucleic acid encoding a multifunctional geranylgeranyl pyrophosphate synthase.

In another aspect, the invention features an isolated nucleic acid having at least 70% sequence identity (e.g., at least 80% or 90%) to the nucleotide sequences of SEQ ID NO:38, or to a fragment of the nucleic acid of SEQ ID NO:38 at least 15 contiguous nucleotides in length. The nucleic acid can encode a β-carotene C4 oxygenase. Canthaxanthin can be made by contacting β-carotene with a polypeptide encoded by such nucleic acids or a polypeptide having an amino acid sequence at least 80% identical to the

amino acid sequence of SEQ ID NO:39. Astaxanthin can be made by contacting zeaxanthin with a polypeptide encoded by such isolated nucleic acids or a polypeptide having an amino acid sequence at least 80% identical to the amino acid sequence of SEQ ID NO:39.

In another aspect, the invention features membranous bacteria that include an exogenous nucleic acid encoding a β-carotene C4 oxygenase, where the β-carotene oxygenase has an amino acid sequence at least 80% identical to the amino acid sequence of SEQ ID NO:39.

In yet another aspect, the invention features a host cell comprising an exogenous nucleic acid, wherein the exogenous nucleic acid includes a nucleic acid sequence encoding one or more polypeptides that catalyze the formation of (3S, 3'S) astaxanthin, wherein the host cell produces CoQ-10 and (3S, 3'S) astaxanthin. A method of making CoQ-10 and (3S, 3'S) astaxanthin at substantially the same time also is featured. The method includes transforming a host cell with a nucleic acid, wherein the nucleic acid includes a nucleic acid sequence that encodes one or more polypeptides, wherein the polypeptides catalyze the formation of (3S, 3'S) astaxanthin; and culturing the host cell under conditions that allow for the production of (3S, 3'S) astaxanthin and CoQ-10. The method further can include transforming the host cell with at least one exogenous nucleic acid, the exogenous nucleic acid encoding one or more polypeptides, wherein the polypeptides catalyze the formation of CoQ-10.

The invention also features isolated nucleic acid having a nucleotide sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:38, and SEQ ID NO:44.

An isolated nucleic acid having at least 90% sequence identity to the nucleotide sequences of SEQ ID NO:44, or to a fragment of the nucleic acid of SEQ ID NO:44 at least 60 contiguous nucleotides in length is featured. Geranylgeranyl pyrophosphate can be made by contacting isopentenyl pyrophosphate and dimethylallyl pyrophosphate with a polypeptide encoded by such a nucleic acid.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those

described herein can be used to practice the invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

DESCRIPTION OF DRAWINGS

FIG 1 is a schematic diagram of the biosynthetic pathway for the production of zeaxanthin and conversion to zeaxanthin di-glucoside.

FIG 2 is a schematic diagram of the *P. stewartii* carotenoid gene operon (6586 bp).

FIG 3 is a chromatogram of astaxanthin production in *P. stewartii::crtW*(*B. aurantiaca*).

DETAILED DESCRIPTION

Nucleic Acid Molecules

The invention features isolated nucleic acids that encode enzymes involved in carotenoid biosynthesis. The nucleic acids of SEQ ID NO:1, 3, 5, 7, 9, and 11 encode zeaxanthin glucosyl transferase (*crtX*), lycopene β-cyclase (*crtY*), geranylgeranyl-pyrophosphate synthase (*crtE*), phytoene desaturase (*crtI*), phytoene synthase (*crtB*) and β-carotene hydroxylase (*crtZ*), respectively. A nucleic acid of the invention can have at least 76% sequence identity, e.g., 78%, 80%, 85%, 90%, 95%, or 99% sequence identity, to the nucleic acid of SEQ ID NO:1, or to fragments of the nucleic acid of SEQ ID NO:1 that are at least about 33 nucleotides in length; at least 78% sequence identity, e.g., 80%, 85%, 90%, 95%, or 99% sequence identity, to the nucleotide sequence of SEQ ID NO:3, or to fragments of the nucleic acid of SEQ ID NO:3 that are at least about 32 nucleotides in length; at least 81% sequence identity, e.g., 82%, 85%, 90%, 95%, or 99% sequence identity, to the nucleotide sequence of SEQ ID NO:5 , or to fragments of the nucleic acid of SEQ ID NO:5 that are at least about 60 nucleotides in length; at least 82% sequence identity, e.g., 83%, 85%, 90%, 95%, or 99% sequence identity, to the nucleotide

sequences of SEQ ID NO:7 or SEQ ID NO:9 , or to fragments of the nucleic acids of SEQ ID NO:7 or SEQ ID NO:9 that are at least about 30 or 23 nucleotides in length, respectively; at least 85% sequence identity, e.g., 86%, 90%, 92%, 95%, or 99% sequence identity, to the nucleotide sequence of SEQ ID NO:11 , or to fragments of the nucleic acid of SEQ ID NO:11 that are at least about 36 nucleotides in length. A nucleic acid of the invention can have at least 60% sequence identity, e.g., at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 99% sequence identity to the nucleotide sequence of SEQ ID NO:38 or to fragments of the nucleic acid of SEQ ID NO:38 that are at least about 15 nucleotides in length. Such a nucleic acid can encode a β-carotene C4 oxygenase (*crtW*). A nucleic acid of the invention also can have at least 90% identity to the nucleotide sequence set forth in SEQ ID NO:44 or to fragments of the nucleic acid of SEQ ID NO:44 that are at least about 60 nucleotides in length. Such a nucleic acid can encode a multifunctional geranylgeranyl pyrophosphate synthase.

Generally, percent sequence identity is calculated by determining the number of matched positions in aligned nucleic acid sequences, dividing the number of matched positions by the total number of aligned nucleotides, and multiplying by 100. A matched position refers to a position in which identical nucleotides occur at the same position in aligned nucleic acid sequences. Percent sequence identity can be determined for any nucleic acid or amino acid sequence as follows. First, a nucleic acid or amino acid sequence is compared to the identified nucleic acid or amino acid sequence using the BLAST 2 Sequences (Bl2seq) program from the stand-alone version of BLASTZ containing BLASTN version 2.0.14 and BLASTP version 2.0.14. This stand-alone version of BLASTZ can be obtained from the University of Wisconsin library as well as at www.fr.com or www.ncbi.nlm.nih.gov. Instructions explaining how to use the Bl2seq program can be found in the readme file accompanying BLASTZ.

Bl2seq performs a comparison between two sequences using either the BLASTN or BLASTP algorithm. BLASTN is used to compare nucleic acid sequences, while BLASTP is used to compare amino acid sequences. To compare two nucleic acid sequences, the options are set as follows: -i is set to a file containing the first nucleic acid sequence to be compared (e.g., C:\seq1.txt); -j is set to a file containing the second nucleic acid sequence to be compared (e.g., C:\seq2.txt); -p is set to blastn; -o is set to any

desired file name (e.g., C:\output.txt); -q is set to –1; -r is set to 2; and all other options are left at their default setting. For example, the following command can be used to generate an output file containing a comparison between two sequences: C:\Bl2seq –i c:\seq1.txt –j c:\seq2.txt –p blastn –o c:\output.txt –q –1 –r 2. To compare two amino acid sequences, the options of Bl2seq are set as follows: -i is set to a file containing the first amino acid sequence to be compared (e.g., C:\seq1.txt); -j is set to a file containing the second amino acid sequence to be compared (e.g., C:\seq2.txt); -p is set to blastp; -o is set to any desired file name (e.g., C:\output.txt); and all other options are left at their default setting. For example, the following command can be used to generate an output file containing a comparison between two amino acid sequences: C:\Bl2seq –i c:\seq1.txt –j c:\seq2.txt –p blastp –o c:\output.txt. If the target sequence shares homology with any portion of the identified sequence, then the designated output file will present those regions of homology as aligned sequences. If the target sequence does not share homology with any portion of the identified sequence, then the designated output file will not present aligned sequences.

Once aligned, a length is determined by counting the number of consecutive nucleotides or amino acid residues from the target sequence presented in alignment with sequence from the identified sequence starting with any matched position and ending with any other matched position. A matched position is any position where an identical nucleotide or amino acid residue is presented in both the target and identified sequence. Gaps presented in the target sequence are not counted since gaps are not nucleotides or amino acid residues. Likewise, gaps presented in the identified sequence are not counted since target sequence nucleotides or amino acid residues are counted, not nucleotides or amino acid residues from the identified sequence.

The percent identity over a particular length is determined by counting the number of matched positions over that length and dividing that number by the length followed by multiplying the resulting value by 100. For example, if (1) a 1000 nucleotide target sequence is compared to the sequence set forth in SEQ ID NO:1, (2) the Bl2seq program presents 200 nucleotides from the target sequence aligned with a region of the sequence set forth in SEQ ID NO: 1 where the first and last nucleotides of that 200 nucleotide region are matches, and (3) the number of matches over those 200 aligned nucleotides is

180, then the 1000 nucleotide target sequence contains a length of 200 and a percent identity over that length of 90 (i.e. 180 ÷ 200 * 100 = 90).

It will be appreciated that a single nucleic acid or amino acid target sequence that aligns with an identified sequence can have many different lengths with each length having its own percent identity. For example, a target sequence containing a 20 nucleotide region that aligns with an identified sequence as follows has many different lengths including those listed in Table 1.

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                    1                   20
Target Sequence:    AGGTCGTGTACTGTCAGTCA (SEQ ID NO:46)
                    |  ||  || | ||||  || || |
Identified Sequence: ACGTGGTGAACTGCCAGTGA (SEQ ID NO:47)
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TABLE 1

Starting Position	Ending Position	Length	Matched Positions	Percent Identity
1	20	20	15	75.0
1	18	18	14	77.8
1	15	15	11	73.3
6	20	15	12	80.0
6	17	12	10	83.3
6	15	10	8	80.0
8	20	13	10	76.9
8	16	9	7	77.8

It is noted that the percent identity value is rounded to the nearest tenth. For example, 78.11, 78.12, 78.13, and 78.14 is rounded down to 78.1, while 78.15, 78.16, 78.17, 78.18, and 78.19 is rounded up to 78.2.. It is also noted that the length value will always be an integer.

Isolated nucleic acid molecules of the invention are at least about 20 nucleotides in length. For example, the nucleic acid molecule can be about 20-30, 22-32, 33-50, 34 to 45, 40-50, 60-80, 62 to 92, 50-100, or greater than 150 nucleotides in length, e.g., 200-300, 300-500, or 500-1000 nucleotides in length. Such fragments, whether protein-encoding or not, can be used as probes, primers, and diagnostic reagents. In some embodiments, the isolated nucleic acid molecules encode a full-length zeaxanthin glucosyl transferase, lycopene β-cyclase, geranylgeranyl pyrophosphate synthase, phytoene desaturase, β-carotene hydroxylase, β-carotene C4 oxygenase, or

multifunctional geranylgeranyl pyrophosphate synthase polypeptide. Nucleic acid molecules can be DNA or RNA, linear or circular, and in sense or antisense orientation.

Isolated nucleic acid molecules of the invention can be produced by standard techniques. As used herein, "isolated" refers to a sequence corresponding to part or all of a gene encoding a zeaxanthin glucosyl transferase, lycopene β-cyclase, geranylgeranyl-pyrophosphate synthase, phytoene desaturase, phytoene synthase, β-carotene hydroxylase, β-carotene C4 oxygenase, or multifunctional geranylgeranyl pyrophosphate synthase polypeptide, or an operon encoding two or more such polypeptides, but free of sequences that normally flank one or both sides of the wild-type gene or the operon in a naturally-occurring genome, e.g., a bacterial genome. The term "isolated" as used herein with respect to nucleic acids also includes any non-naturally-occurring nucleic acid sequence since such non-naturally-occurring sequences are not found in nature and do not have immediately contiguous sequences in a naturally-occurring genome.

An isolated nucleic acid can be, for example, a DNA molecule, provided one of the nucleic acid sequences normally found immediately flanking that DNA molecule in a naturally-occurring genome is removed or absent. Thus, an isolated nucleic acid includes, without limitation, a DNA molecule that exists as a separate molecule (e.g., a cDNA or genomic DNA fragment produced by PCR or restriction endonuclease treatment) independent of other sequences as well as recombinant DNA that is incorporated into a vector, an autonomously replicating plasmid, a virus (e.g., a retrovirus, adenovirus, or herpes virus), or into the genomic DNA of a prokaryote or eukaryote. In addition, an isolated nucleic acid can include an engineered nucleic acid such as a recombinant DNA molecule that is part of a hybrid or fusion nucleic acid. A nucleic acid existing among hundreds to millions of other nucleic acids within, for example, cDNA libraries or genomic libraries, or gel slices containing a genomic DNA restriction digest, is not to be considered an isolated nucleic acid.

Isolated nucleic acids within the scope of the invention can be obtained using any method including, without limitation, common molecular cloning and chemical nucleic acid synthesis techniques. For example, polymerase chain reaction (PCR) techniques can be used to obtain an isolated nucleic acid containing a nucleic acid sequence sharing identity with the sequences set forth in SEQ ID NOs: 1, 3, 5, 7, 9, 11, 38, or 44. PCR

refers to a procedure or technique in which target nucleic acids are amplified. Sequence information from the ends of the region of interest or beyond typically is employed to design oligonucleotide primers that are identical in sequence to opposite strands of the template to be amplified. PCR can be used to amplify specific sequences from DNA as well as RNA, including sequences from total genomic DNA or total cellular RNA. Primers are typically 14 to 40 nucleotides in length, but can range from 10 nucleotides to hundreds of nucleotides in length. General PCR techniques are described, for example in PCR Primer: A Laboratory Manual, Ed. by Dieffenbach, C. and Dveksler, G., Cold Spring Harbor Laboratory Press, 1995. When using RNA as a source of template, reverse transcriptase can be used to synthesize complimentary DNA (cDNA) strands.

Isolated nucleic acids of the invention also can be chemically synthesized, either as a single nucleic acid molecule or as a series of oligonucleotides. For example, one or more pairs of long oligonucleotides (e.g., >100 nucleotides) can be synthesized that contain the desired sequence, with each pair containing a short segment of complementary (e.g., about 15 nucleotides) DNA such that a duplex is formed when the oligonucleotide pair is annealed. DNA polymerase is used to extend the oligonucleotides, resulting in a double-stranded nucleic acid molecule per oligonucleotide pair, which then can be ligated into a vector.

Isolated nucleic acids of the invention also can be obtained by mutagenesis. For example, an isolated nucleic acid that shares identity with a sequence set forth in SEQ ID NO: 1, 3, 5, 7, 9, 11, 38, or 44 can be mutated using common molecular cloning techniques (e.g., site-directed mutagenesis). Possible mutations include, without limitation, deletions, insertions, and substitutions, as well as combinations of deletions, insertions, and substitutions. Alignments of nucleic acids of the invention with other known sequences encoding carotenoid enzymes can be used to identify positions to modify. For example, alignment of the nucleotide sequence of SEQ ID NO:5 with other nucleic acids encoding geranyl geranyl pyrophosphate synthases (e.g., from *Erwinia uredovora*) provides guidance as to which nucleotides can be substituted, which nucleotides can be deleted, and at which positions nucleotides can be inserted.

In addition, nucleic acid and amino acid databases (e.g., GenBank®) can be used to obtain an isolated nucleic acid within the scope of the invention. For example, any

nucleic acid sequence having homology to a sequence set forth in SEQ ID NO: 1, 3, 5, 7, 9, 11, 38, or 44, or any amino acid sequence having homology to a sequence set forth in SEQ ID NO: 2, 4, 6, 8, 10, 12, 39, or 45 can be used as a query to search GenBank®.

Furthermore, nucleic acid hybridization techniques can be used to obtain an isolated nucleic acid within the scope of the invention. Briefly, any nucleic acid having some homology to a sequence set forth in SEQ ID NO: 1, 3, 5, 7, 9, 11, 38, or 44 can be used as a probe to identify a similar nucleic acid by hybridization under conditions of moderate to high stringency. Moderately stringent hybridization conditions include hybridization at about 42°C in a hybridization solution containing 25 mM KPO_4 (pH 7.4), 5X SSC, 5X Denhart's solution, 50 μg/mL denatured, sonicated salmon sperm DNA, 50% formamide, 10% Dextran sulfate, and 1-15 ng/mL probe (about $5x10^7$ cpm/μg), and wash steps at about 50°C with a wash solution containing 2X SSC and 0.1% SDS. For high stringency, the same hybridization conditions can be used, but washes are performed at about 65°C with a wash solution containing 0.2X SSC and 0.1% SDS.

Once a nucleic acid is identified, the nucleic acid then can be purified, sequenced, and analyzed to determine whether it is within the scope of the invention as described herein. Hybridization can be done by Southern or Northern analysis to identify a DNA or RNA sequence, respectively, that hybridizes to a probe. The probe can be labeled with biotin, digoxygenin, an enzyme, or a radioisotope such as ^{32}P or ^{35}S. The DNA or RNA to be analyzed can be electrophoretically separated on an agarose or polyacrylamide gel, transferred to nitrocellulose, nylon, or other suitable membrane, and hybridized with the probe using standard techniques well known in the art. See, for example, sections 7.39-7.52 of Sambrook *et al.*, (1989) Molecular Cloning, second edition, Cold Spring harbor Laboratory, Plainview, NY.

Polypeptides

The present invention also features isolated zeaxanthin glucosyl transferase (SEQ ID NO:2), lycopene β-cyclase (SEQ ID NO:4), geranylgeranyl pyrophosphate synthase (SEQ ID NO:6), phytoene desaturase (SEQ ID NO:8), phytoene synthase (SEQ ID NO:10), and β-carotene hydroxylase (SEQ ID NO:12) polypeptides. In addition, the invention features isolated β-carotene C4 oxygenase polypeptides (SEQ ID NO:39) and

multifunctional geranylgeranyl pyrophosphate synthase polypeptides (SEQ ID NO:45). A polypeptide of the invention can have at least 75% sequence identity, e.g., 80%, 85%, 90%, 95%, or 99% sequence identity, to the amino acid sequence of SEQ ID NO:2 or to fragments thereof; at least 83% sequence identity, e.g., 85%, 90%, 95%, or 99% sequence identity, to the amino acid sequence of SEQ ID NO:4 or to fragments thereof; at least 85% sequence identity, e.g., 90%, 95%, or 99% sequence identity, to the amino acid sequence of SEQ ID NO:6 or to fragments thereof; at least 90% sequence identity, e.g., 90%, 92%, 95%, or 99% sequence identity, to the amino acid sequence of SEQ ID NO:8 or to fragments thereof; at least 89% sequence identity, e.g., 90%, 95%, or 99% sequence identity, to the amino acid sequence of SEQ ID NO:10 or to fragments thereof; at least 90% sequence identity, e.g., 95%, or 99% sequence identity, to the amino acid sequence of SEQ ID NO:12 or to fragments thereof; at least 60% sequence identity, e.g., 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 99% sequence identity, to the amino acid sequence of SEQ ID NO:39 or to fragments thereof; or at least 90% sequence identity, e.g., 95% or 99% sequence identity, to the amino acid sequence set forth in SEQ ID NO:45 or to fragments thereof. Percent sequence identity can be determined as described above for nucleic acid molecules.

An "isolated polypeptide" has been separated from cellular components that naturally accompany it. Typically, the polypeptide is isolated when it is at least 60% (e.g., 70%, 80%, 90%, 95%, or 99%), by weight, free from proteins and naturally-occurring organic molecules that are naturally associated with it. In general, an isolated polypeptide will yield a single major band on a non-reducing polyacrylamide gel.

The term "polypeptide" includes any chain of amino acids, regardless of length or post-translational modification. Polypeptides that have identity to the amino acid sequences of SEQ ID NO:2, 4, 6, 8, 10, 12, 39, or 45 can retain the function of the enzyme (see FIG 1 for a schematic of the carotenoid biosynthesis pathway). For example, geranylgeranyl pyrophosphate synthase can produce geranylgeranyl pyrophosphate (GGPP) by condensing together isopentenyl pyrophosphate (IPP) with farnesyl pyrophosphate (FPP). Phytoene synthase can produce phytoene by condensing together two molecules of GGPP. Phytoene desaturase can perform four successive desaturations on phytoene to form lycopene. Lycopene β-cyclase can perform two

successive cyclization reactions on lycopene to form β-carotene. β-carotene hydroxylase can perform two successive hydroxylation reactions on β-carotene to form zeaxanthin. Alternatively, β-carotene hydroxylase can perform two successive hydroxylation reactions on canthaxanthin to form astaxanthin. Zeaxanthin glucosyl transferase can add one or two glucose or other sugar moieties to zeaxanthin to form zeaxanthin monoglycoside or diglycoside, respectively. β-carotene C4 oxygenase can convert the methylene groups at the C4 and C4' positions of the β-carotene or zeaxanthin to form canthaxanthin or astaxanthin, respectively. Multifunctional geranylgeranyl pyrophosphate synthase can directly convert 3 IPP molecules and 1 dimethylallyl pyrophosphate (DMAPP) molecule to 1 GGPP molecule.

In general, conservative amino acid substitutions, i.e., substitutions of similar amino acids, are tolerated without affecting protein function. Similar amino acids are those that are similar in size and/or charge properties. Families of amino acids with similar side chains are known. These families include amino acids with basic side chains (e.g., lysine, arginine, or histidine), acidic side chains (e.g., aspartic acid or glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, or cysteine), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, or tryptophan), β-branched side chains (e.g., threonine, valine, or isoleucine), and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, or histidine).

Mutagenesis also can be used to alter a nucleic acid such that activity of the polypeptide encoded by the nucleic acid is altered (e.g., to increase production of a particular carotenoid). For example, error-prone PCR (e.g., (GeneMorph PCR Mutagenesis Kit; Stratagene Inc. La Jolla, CA; Catalog # 600550; Revision #090001) can be used to mutagenize the *B. aurantiaca crtW* gene (SEQ ID NO:38) to increase the relative amount of di-keto carotenoid (e.g. astaxanthin (3,3'-dihydroxy-β,β-carotene-4,4'-dione) or canthaxanthin (β,β-carotene-4,4'-dione)) relative to mono-keto carotenoid (e.g. echinone (β,β-carotene-4-one) or adonixanthin (3,3'-dihydroxy-β,β-carotene-4-one)) that is produced. In general, the nucleic acid to be mutagenized can be cloned into a vector such as pCR-Blunt II-TOPO (Clontech; Palo Alto, CA) and used as a template for error-prone PCR. For purposes of directed evolution, mutation frequencies of 2-7 nucleotides /

Kbp template (1-4 amino acids mutations / 333 Amino acids) generally are desired. Mutation frequency can be lowered or raised by increasing or decreasing the template concentration, respectively. PCR can be performed according to manufacturer's recommendations. Mutagenized nucleic acid is ligated into an expression vector, which is used to transform a host, and activity of the expressed protein is assessed. For example, in the case of the *crtW* gene, electrocompetent *P. stewartii* (ATCC 8200) cells can be prepared and transformed as described herein, and resulting individual colonies can be screened by visual inspection for a phenotypic change from bright yellow pigmentation (production of zeaxanthin), yellow orange (production of mono-keto carotenoid) or reddish-orange (production of di-keto carotenoid). Production of increased amounts of astaxanthin can be confirmed by HPLC/MS.

Isolated polypeptides of the invention can be obtained, for example, by extraction from a natural source (e.g., a plant or bacteria cell), chemical synthesis, or by recombinant production in a host. For example, a polypeptide of the invention can be produced by ligating a nucleic acid molecule encoding the polypeptide into a nucleic acid construct such as an expression vector, and transforming a bacterial or eukaryotic host cell with the expression vector. In general, nucleic acid constructs include expression control elements operably linked to a nucleic acid sequence encoding a polypeptide of the invention (e.g., zeaxanthin glucosyl transferase, lycopene β-cyclase, geranylgeranyl pyrophosphate synthase, phytoene desaturase, phytoene synthase, β-carotene hydroxylase, β-carotene C4 oxygenase, or multifunctional geranylgeranyl pyrophosphate synthase polypeptides). Expression control elements do not typically encode a gene product, but instead affect the expression of the nucleic acid sequence. As used herein, "operably linked" refers to connection of the expression control elements to the nucleic acid sequence in such a way as to permit expression of the nucleic acid sequence. Expression control elements can include, for example, promoter sequences, enhancer sequences, response elements, polyadenylation sites, or inducible elements. Non-limiting examples of promoters include the *puf* promoter from *Rhodobacter sphaeroides* (GenBank Accession No. E13945), the *nifHDK* promoter from *R. sphaeroides* (GenBank Accession No. AF031817), and the *fliK* promoter from *R. sphaeroides* (GenBank Accession No. U86454).

In bacterial systems, a strain of *E. coli* such as DH10B or BL-21 can be used. Suitable *E. coli* vectors include, but are not limited to, pUC18, pUC19, the pGEX series of vectors that produce fusion proteins with glutathione S-transferase (GST), and pBluescript series of vectors. Transformed *E. coli* are typically grown exponentially then stimulated with isopropylthiogalactopyranoside (IPTG) prior to harvesting. In general, fusion proteins produced from the pGEX series of vectors are soluble and can be purified easily from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites such that the cloned target gene product can be released from the GST moiety.

In eukaryotic host cells, a number of viral-based expression systems can be utilized to express polypeptides of the invention. A nucleic acid encoding a polypeptide of the invention can be cloned into, for example, a baculoviral vector such as pBlueBac (Invitrogen, San Diego, CA) and then used to co-transfect insect cells such as *Spodoptera frugiperda* (Sf9) cells with wild-type DNA from *Autographa californica* multiply enveloped nuclear polyhedrosis virus (AcMNPV). Recombinant viruses producing polypeptides of the invention can be identified by standard methodology. Alternatively, a nucleic acid encoding a polypeptide of the invention can be introduced into a SV40, retroviral, or vaccinia based viral vector and used to infect suitable host cells.

A polypeptide within the scope of the invention can be "engineered" to contain an amino acid sequence that allows the polypeptide to be captured onto an affinity matrix. For example, a tag such as c-myc, hemagglutinin, polyhistidine, or Flag™ tag (Kodak) can be used to aid polypeptide purification. Such tags can be inserted anywhere within the polypeptide including at either the carboxyl or amino termini. Other fusions that could be useful include enzymes that aid in the detection of the polypeptide, such as alkaline phosphatase.

Agrobacterium-mediated transformation, electroporation and particle gun transformation can be used to transform plant cells. Illustrative examples of transformation techniques are described in U.S. Patent No. 5,204,253 (particle gun) and U.S. Patent No. 5,188,958 (*Agrobacterium*). Transformation methods utilizing the Ti and Ri plasmids of *Agrobacterium spp.* typically use binary type vectors. Walkerpeach, C. et

al., in Plant Molecular Biology Manual, S. Gelvin and R. Schilperoort, eds., Kluwer Dordrecht, C1:1-19 (1994). If cell or tissue cultures are used as the recipient tissue for transformation, plants can be regenerated from transformed cultures by techniques known to those skilled in the art.

Engineered cells

Any cell containing an isolated nucleic acid within the scope of the invention is itself within the scope of the invention. This includes, without limitation, prokaryotic cells such as *R. sphaeroides* cells and eukaryotic cells such as plant, yeast, and other fungal cells. It is noted that cells containing an isolated nucleic acid of the invention are not required to express the isolated nucleic acid. In addition, the isolated nucleic acid can be integrated into the genome of the cell or maintained in an episomal state. In other words, cells can be stably or transiently transfected with an isolated nucleic acid of the invention.

Any method can be used to introduce an isolated nucleic acid into a cell. In fact, many methods for introducing nucleic acid into a cell, whether *in vivo* or *in vitro*, are well known to those skilled in the art. For example, calcium phosphate precipitation, conjugation, electroporation, heat shock, lipofection, microinjection, and viral-mediated nucleic acid transfer are common methods that can be used to introduce nucleic acid molecules into a cell. In addition, naked DNA can be delivered directly to cells *in vivo* as describe elsewhere (U.S. Patent Nos. 5,580,859 and 5,589,466). Furthermore, nucleic acid can be introduced into cells by generating transgenic animals.

Any method can be used to identify cells that contain an isolated nucleic acid within the scope of the invention. For example, PCR and nucleic acid hybridization techniques such as Northern and Southern analysis can be used. In some cases, immunohistochemistry and biochemical techniques can be used to determine if a cell contains a particular nucleic acid by detecting the expression of a polypeptide encoded by that particular nucleic acid. For example, the polypeptide of interest can be detected with an antibody having specific binding affinity for that polypeptide, which indicates that that cell not only contains the introduced nucleic acid but also expresses the encoded polypeptide. Enzymatic activities of the polypeptide of interest also can be detected or an

end product (e.g., a particular carotenoid) can be detected as an indication that the cell contains the introduced nucleic acid and expresses the encoded polypeptide from that introduced nucleic acid.

The cells described herein can contain a single copy, or multiple copies (e.g., about 5, 10, 20, 35, 50, 75, 100 or 150 copies), of a particular exogenous nucleic acid. All non-naturally-occurring nucleic acids are considered an exogenous nucleic acid once introduced into the cell. The term "exogenous" as used herein with reference to a nucleic acid and a particular cell refers to any nucleic acid that does not originate from that particular cell as found in nature. Nucleic acid that is naturally-occurring also can be exogenous to a particular cell. For example, an entire operon that is isolated from a bacteria is an exogenous nucleic acid with respect to a second bacteria once that operon is introduced into the second bacteria. For example, a bacterial cell (e.g., *Rhodobacter*) can contain about 50 copies of an exogenous nucleic acid of the invention. In addition, the cells described herein can contain more than one particular exogenous nucleic acid. For example, a bacterial cell can contain about 50 copies of exogenous nucleic acid X as well as about 75 copies of exogenous nucleic acid Y. In these cases, each different nucleic acid can encode a different polypeptide having its own unique enzymatic activity. For example, a bacterial cell can contain two different exogenous nucleic acids such that a high level of astaxanthin or other carotenoid is produced. In addition, a single exogenous nucleic acid can encode one or more polypeptides. For example, a single nucleic acid can contain sequences that encode three or more different polypeptides.

Microorganisms that are suitable for producing carotenoids may or may not naturally produce carotenoids, and include prokaryotic and eukaryotic microorganisms, such as bacteria, yeast, and fungi. In particular, yeast such as *Phaffia rhodozyma* (*Xanthophyllomyces dendrorhous*), *Candida utilis*, and *Saccharomyces cerevisiae*, fungi such as *Neurospora crassa*, *Phycomyces blakesleeanus*, *Blakeslea trispora*, and *Aspergillus sp*, Archaeabacteria such as *Halobacterium salinarium*, and Eubacteria including *Pantoea* species (formerly called *Erwinia*) such as *Pantoea stewartii* (e.g., ATCC Accession #8200), flavobacteria species such as *Xanthobacter autotrophicus* and *Flavobacterium multivorum*, *Zymonomonas mobilis*, *Rhodobacter* species such as *R. sphaeroides* and *R. capsulatus*, *E. coli*, and *E. vulneris* can be used. Other examples of

bacteria that may be used include bacteria in the genus *Sphingomonas* and Gram negative bacteria in the α-subdivision, including, for example, *Paracoccus*, *Azotobacter*, *Agrobacterium*, and *Erythrobacter*. Eubacteria, and especially *R. sphaeroides* and *R. capsulatus*, are particularly useful. *R. sphaeroides* and *R. capsulatus* naturally produce certain carotenoids and grows on defined media. Such *Rhodobacter* species also are non-pyrogenic, minimizing health concerns about use in nutritional supplements. In some embodiments, it can be useful to produce carotenoids in plants and algae such as *Zea mays*, *Brassica napus*, *Lycopersicon esculentum*, *Tagetes erecta*, *Haematococcus pluvialis*, *Dunaliella salina*, *Chlorella protothecoides*, and *Neospongiococcum excentrum*.

It is noted that bacteria can be membranous or non-membranous bacteria. The term "membranous bacteria" as used herein refers to any naturally-occurring, genetically modified, or environmentally modified bacteria having an intracytoplasmic membrane. An intracytoplasmic membrane can be organized in a variety of ways including, without limitation, vesicles, tubules, thylakoid-like membrane sacs, and highly organized membrane stacks. Any method can be used to analyze bacteria for the presence of intracytoplasmic membranes including, without limitation, electron microscopy, light microscopy, and density gradients. See, e.g., Chory et al., (1984) J. Bacteriol., 159:540-554; Niederman and Gibson, Isolation and Physiochemical Properties of Membranes from Purple Photosynthetic Bacteria. In: The Photosynthetic Bacteria, Ed. By Roderick K. Clayton and William R. Sistrom, Plenum Press, pp. 79-118 (1978); and Lueking et al., (1978) J. Biol. Chem., 253: 451-457. Examples of membranous bacteria that can be used include, without limitation, Purple Non-Sulfur Bacteria, including bacteria of the Rhodospirillaceae family such as those in the genus *Rhodobacter* (e.g., *R. sphaeroides* and *R. capsulatus*), the genus *Rhodospirillum*, the genus *Rhodopseudomonas*, the genus *Rhodomicrobium*, and the genus *Rhodophila*. The term "non-membranous bacteria" refers to any bacteria lacking intracytoplasmic membrane. Membranous bacteria can be highly membranous bacteria. The term "highly membranous bacteria" as used herein refers to any bacterium having more intracytoplasmic membrane than *R. sphaeroides* (ATCC 17023) cells have after the *R. sphaeroides* (ATCC 17023) cells have been (1) cultured chemoheterotrophically under aerobic condition for four days, (2) cultured

chemoheterotrophically under anaerobic for four hours, and (3) harvested. Aerobic culture conditions include culturing the cells in the dark at 30°C in the presence of 25% oxygen. Anaerobic culture conditions include culturing the cells in the light at 30°C in the presence of 2% oxygen. After the four hour anaerobic culturing step, the *R. sphaeroides* (ATCC 17023) cells are harvested by centrifugation and analyzed.

Nucleic acids of the invention can be expressed in microorganisms so that detectable amounts of carotenoids are produced. As used herein, "detectable" refers to the ability to detect the carotenoid and any esters or glycosides thereof using standard analytical methodology. In general, carotenoids can be extracted with an organic solvent such as acetone or methanol and detected by an absorption scan from 400-500 nm in the same organic solvent. In some cases, it is desirable to back-extract with a second organic solvent, such as hexane. The maximal absorbance of each carotenoid depends on the solvent that it is in. For example, in acetone, the maximal absorbance of lutein is at 451 nm, while maximal absorbance of zeaxanthin is at 454 nm. In hexane, the maximal absorbance of lutein and zeaxanthin is 446 nm and 450 nm, respectively. High performance liquid chromatography coupled to mass spectrometry also can be used to detect carotenoids. Two reverse phase columns that are connected in series can be used with a solvent gradient of water and acetone. The first column can be a C30 specialty column designed for carotenoid separation (e.g., YMCä Carotenoid S3m; 2.0 x 150 mm, 3mm particle size; Waters Corporation, PN CT99S031502WT) followed by a C8 Xterraä MS column (e.g., Xterraä MS C8; 2.1 x 250 mm, 5mm particle size; Waters Corporation, PN 186000459).

Detectable amounts of carotenoids include 10μg/g dry cell weight (dcw) and greater. For example, about 10 to 100,000μg/g dcw, about 100 to 60,000μg/g dcw, about 500 to 30,000μg/g dcw, about 1000 to 20,000 μg/g dcw, about 5,000 to 55,000 μg/g dcw, or about 30,000 μg/g dcw to about 55,000 μg/g dcw. With respect to algae or other plants or organisms that produce a particular carotenoid, such as astaxanthin, β-carotene, lycopene, or zeaxanthin, "detectable amount" of carotenoid is an amount that is detectable over the endogenous level in the plant or organism.

Depending on the microorganism and the metabolites present within the microorganism, one or more of the following enzymes may be expressed in the

microorganism: geranylgeranyl pyrophosphate synthase, phytoene synthase, phytoene desaturase, lycopene β cyclase, lycopene ε cyclase, zeaxanthin glycosyl transferase, β-carotene hydroxylase, β-carotene C-4 ketolase, and multifunctional geranylgeranyl pyrophosphate synthase. Suitable nucleic acids encoding these enzymes are described above. Also, see, for example, Genbank Accession No. Y15112 for the sequence of carotenoid biosynthesis genes of *Paracoccus marcusii*; Genbank Accession No. D58420 for the carotenoid biosynthesis genes of *Agrobacterium aurantiacum*; Genbank Accession No. M87280 M99707 for the sequence of carotenoid biosynthesis genes of *Erwinia herbicola*; and Genbank Accession No. U62808 for carotenoid biosynthesis genes of *Flavobacterium* sp. Strain R1534.

For example, to produce lycopene in a microorganism that naturally produces neurosporene, such as *Rhodobacter*, an exogenous nucleic acid encoding phytoene desaturase can be expressed, e.g., a phytoene desaturase of the invention, and lycopene can be detected using standard methodology. Expression of additional carotenoid genes in such an engineered cell will allow for production of additional carotenoids. For example, expression of a lycopene β-cyclase in such an engineered cell allows production of detectable amounts of β-carotene, while further expression of a β-carotene hydroxylase allows production of another carotenoid, zeaxanthin. β-carotene and zeaxanthin can be detected using standard methodology and are distinguished by mobility on an HPLC column. Zeaxanthin diglucoside can be produced by further expression of zeaxanthin glucosyl transferase (*crtX*) in an organism that produces zeaxanthin.

Alternatively, canthaxanthin can be produced in organisms that produce phytoene by expression of phytoene desaturase, lycopene β-cyclase, and β-carotene C4 oxygenase, an enzyme that converts the methylene groups at the C4 and C4' positions of the carotenoid to ketone groups. The β-carotene C4 oxygenase from, e.g., *Agrobacterium aurantiacum* or *Haematococcus pluvialis* can be used. See, GenBank Accession Nos. 1136630 and X86782 for a description of the nucleotide and amino acid sequences of the *A. aurantiacum* and *H. pluvialis* enzymes, respectively. The β-carotene C4 oxygenase from *Brevundimonas aurantiaca* also can be used. See, Example 2 for a description of the nucleotide and amino acid sequences. In organisms that do not naturally produce carotenoids, additional enzymes are required for production of canthaxanthin.

Geranylgeranyl pyrophosphate synthase and phytoene synthase can be expressed such that the necessary precursors for canthaxanthin synthesis are present.

Astaxanthin also can be produced in microorganisms that naturally produce carotenoids. For example, a *Rhodobacter* cell can be engineered such that phytoene desaturase, lycopene β-cyclase, β-carotene hydroxylase, and β-carotene C4 oxygenase are expressed and detectable amounts of astaxanthin are produced. Such an organism also can express an enzyme that can modify the 3 or 3' hydroxyl groups of astaxanthin with chemical groups such as glucose (e.g., to produce astaxanthin diglucoside), other sugars, or fatty acids. In addition, a *P. stewartii* cell can be engineered such that β-carotene C4 oxygenase is expressed and detectable amounts of astaxanthin are produced. Astaxanthin can be detected as described above, and has maximal absorbance at 480 nm in acetone.

Yields of astaxanthin and other carotenoids can be increased by expression of a multifunctional geranylgeranyl pyrophosphate synthase, such as that from *S. shibatae* (SEQ ID NO:45) or an Archaebacterial gene from *Archaeoglobus fulgidus* (GenBank Accession No. AF120272), in the engineered microorganism. The archaebacteria GGPPS gene is a homolog of the endogenous *Rhodobacter* gene and encodes an enzyme that directly converts 3 IPP molecules and 1 DMAPP molecule to 1 GGPPS molecule, thereby reducing branching of the carotenoid pathway and eliminating production of other less desirable isoprenoids. Further reductions in less desirable metabolites can be obtained by eliminating endogenous bacteriochlorophyll biosynthesis, which redirects flow into carotenoid biosynthesis. For example, the *bchO*, *bchD*, and *bchI* genes can be deleted and/or replaced with an Archaebacterial GGPPS gene. Additional increases in yield can be obtained by deletion of the endogenous *crtE* gene or the endogenous *crtC*, *crtD*, *crtE*, *crtA*, *crtI*, and *crtF* genes.

Common mutagenesis or knock-out technology can be used to delete endogenous genes. Alternatively, antisense technology can be used to reduce enzymatic activity. For example, a *R. sphaeroides* cell can be engineered to contain a cDNA that encodes an antisense molecule that prevents an enzyme from being made. The term "antisense molecule" as used herein encompasses any nucleic acid that contains sequences that correspond to the coding strand of an endogenous polypeptide. An antisense molecule also can have flanking sequences (e.g., regulatory sequences). Thus, antisense molecules

can be ribozymes or antisense oligonucleotides. A ribozyme can have any general structure including, without limitation, hairpin, hammerhead, or axhead structures, provided the molecule cleaves RNA.

Control of the Ratio of Carotenoids

The amount of particular carotenoids, such as astaxanthin to canthaxanthin, or astaxanthin to zeaxanthin, can be controlled by expression of carotenoid genes from an inducible promoter or by use of constitutive promoters of different strengths. As used herein, "inducible" refers to both up-regulation and down regulation. An inducible promoter is a promoter that is capable of directly or indirectly activating transcription of one or more DNA sequences or genes in response to an inducer. In the absence of an inducer, the DNA sequences or genes will not be transcribed. The inducer can be a chemical agent such as a protein, metabolite, growth regulator, phenolic compound, or a physiological stress imposed directly by heat, cold, salt, or toxic elements, or indirectly through the action of a pathogen or disease agent such as a virus. The inducer also can be an illumination agent such as light, darkness and light's various aspects, which include wavelength, intensity, fluorescence, direction, and duration. Examples of inducible promoters include the lac system and the tetracycline resistance system from *E. coli*. In one version of the lac system, expression of lac operator-linked sequences is constitutively activated by a lacR-VP16 fusion protein and is turned off in the presence of IPTG. In another version of the lac system, a lacR-VP16 variant is used that binds to lac operators in the presence of IPTG, which can be enhanced by increasing the temperature of the cells.

Components of the tetracycline (Tc) resistance system also can be used to regulate gene expression. For example, the Tet repressor (TetR), which binds to tet operator sequences in the absence of tetracycline and represses gene transcription, can be used to repress transcription from a promoter containing tet operator sequences. TetR also can be fused to the activation domain of VP 16 to create a tetracycline-controlled transcriptional activator (tTA), which is regulated by tetracycline in the same manner as TetR, i.e., tTA binds to tet operator sequences in the absence of tetracycline but not in the presence of

tetracycline. Thus, in this system, in the continuous presence of Tc, gene expression is repressed, and to induce transcription, Tc is removed.

Alternative methods of controlling the ratio of carotenoids include using enzyme inhibitors to regulate the activity levels of particular enzymes.

Production of Carotenoids

Carotenoids can be produced *in vitro* or *in vivo*. For example, one or more polypeptides of the invention can be contacted with an appropriate substrate or combination of substrates to produce the desired carotenoid (e.g., astaxanthin). See, FIG. 1 for a schematic of the carotenoid biosynthetic pathway.

A particular carotenoid (e.g., astaxanthin, lycopene, β-carotene, lutein, zeaxanthin, zeaxanthin diglucoside, or canthaxanthin) also can be produced by providing an engineered microorganism and culturing the provided microorganism with culture medium such that the carotenoid is produced. In general, the culture media and/or culture conditions are such that the microorganisms grow to an adequate density and produce the desired compound efficiently. For large-scale production processes, the following methods can be used. First, a large tank (e.g., a 100 gallon, 200 gallon, 500 gallon, or more tank) containing appropriate culture medium with, for example, a glucose carbon source is inoculated with a particular microorganism. After inoculation, the microorganisms are incubated to allow biomass to be produced. Once a desired biomass is reached, the broth containing the microorganisms can be transferred to a second tank. This second tank can be any size. For example, the second tank can be larger, smaller, or the same size as the first tank. Typically, the second tank is larger than the first such that additional culture medium can be added to the broth from the first tank. In addition, the culture medium within this second tank can be the same as, or different from, that used in the first tank. For example, the first tank can contain medium with xylose, while the second tank contains medium with glucose.

Once transferred, the microorganisms can be incubated to allow for the production of the desired carotenoid. Once produced, any method can be used to isolate the desired compound. For example, if the microorganism releases the desired carotenoid into the broth, then common separation techniques can be used to remove the biomass

from the broth, and common isolation procedures (e.g., extraction, distillation, and ion-exchange procedures) can be used to obtain the carotenoid from the microorganism-free broth. In addition, the desired carotenoid can be isolated while it is being produced, or it can be isolated from the broth after the product production phase has been terminated. If the microorganism retains the desired carotenoid, the biomass can be collected and the carotenoid can be released by treating the biomass or the carotenoid can be extracted directly from the biomass. Extracted carotenoid can be formulated as a nutraceutical. As used herein, a nutraceutical refers to a compound(s) that can be incorporated into a food, tablet, powder, or other medicinal form that, upon ingestion by a subject, provides a specific medical or physiological benefit to the subject.

Alternatively, the biomass can be collected and dried, without extracting the carotenoids. The biomass then can be formulated for human consumption (e.g., as a dietary supplement) or as an animal feed (e.g., for companion animals such as dogs, cats, and horses, or for production animals). For example, the biomass can be formulated for consumption by poultry such as chickens and turkeys, or by cattle, pigs, and sheep. Feeding of such compositions may increase yield of breast meat in poultry and may increase weight gain in other farm animals. In addition, the carotenoids may increase shelf-life of meat products due to the increased antioxidant protection afforded by the carotenoids. The biomass also can be formulated for use in aquaculture. For example, biomass that includes an engineered microorganism that is producing, e.g., astaxanthin and/or canthaxanthin, can be fed to fish or crustaceans to pigment the flesh or carapace, respectively. Such a composition is particularly useful for feeding to fish such as salmon, trout, sea breem, or snapper, or crustaceans such as shrimp, lobster, and crab.

One or more components can be added to the biomass before or after drying, including vitamins, other carotenoids, antioxidants such as ethoxyquin, vitamin E, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), or ascorbyl palmitate, vegetable oils such as corn oil, safflower oil, sunflower oil, or soybean oil, and an edible emulsifier, such as soy bean lecithin or sorbitan esters. Addition of antioxidants and vegetable oils can help prevent degradation of the carotenoid during processing (e.g., drying), shipment, and storage of the composition.

The invention will be further described in the following examples, which do not limit the scope of the invention described in the claims.

EXAMPLES

Example 1 - Cloning of the zeaxanthin gene cluster from *Pantoea stewartii*:

Genomic DNA from *P. stewartii* was isolated and digested with restriction enzymes to yield genomic DNA fragments approximately 8-10 kB in size. These genomic DNA fragments were ligated into a vector cut with the same restriction enzyme, and electroporated into electrocompetent *E. coli*. Transformant colonies were individually picked and transferred onto fresh solid media with the appropriate antibiotic selection (ampicillin/ampicillin substitute). It was thought that *E. coli* colonies containing the *P. stewartii* carotenoid genes would appear yellow in color due to the production of zeaxanthin pigment or red due to the production of lycopene. Although at least 2000 ampicillin resistant *E. coli* transformants were screened, none of the colonies were found to contain the *P. stewartii* carotenoid genes.

Instead, a second, PCR based method was used to identify and sequence the carotenoid (crt) gene cluster from *P. stewartii* genomic DNA. Degenerate primers were designed based on homologous regions identified in the crt genes from *Erwinia herbicola* and *Erwinia uredovora*. Table 2 provides the position of the crt genes in *E. herbicola* and *E. uredovora*.

TABLE 2

Position of crt genes in *E. herbicola* and *E. uredovora*

Gene name	Start of Gene (nucleotide #)		End of Gene (nucleotide #)	
	E. herbicola	*E. uredovora*	*E. herbicola*	*E. uredovora*
CrtE	3535	198	4458	1133
Orf-6	4521		5564	
CrtX	5561	1143	6802	2438
CrtY	6799	2422	7959	3570
CrtI	7956	3582	9434	5060
CrtB	9431	5096	10360	5986
CrtZ	10826 (complement)	6452 (complement)	10296 complement	5925 (complement)
Orf-12	12127 complement		10916 complement	

The following primers were designed (Table 3) and used in various combinations to yield PCR products of varying lengths. *P. stewartii* genomic DNA was used as template.

TABLE 3
Sequences of Degenerate Primers

Primer Name	Primer Sequence	SEQ ID NO
P.s.BCHy1	5'-ATYATGCACGGCTGGGGWTGGSGMTGGCA - 3'	13
P.s. BCHy2	5' - GGCCARCGYTGATGCACCAGMCCGTCRTGCA - 3'	14
P.s.PS1	5' - CTGATGCTCTAYGCCTGGTGCCGCCA - 3'	15
P.s.PS2	5' - TCGCGRGCRATRTTSGTCARCTG - 3'	16
P.s.LBC1	5' - ATBMTSATGGAYGCSACSGT - 3'	17
P.s.LBC2	5' - YTRATCGARGAYACGCRCTA - 3'	18
P.s.LBC3	5' - RSGGCAGYGAATAGCCRGTG - 3'	19
P.s.LBC4	5' - AACAGCATSCGRTTCAGCAKGCGSA - 3'	20
P.s.PD5	5' - CCGACGGTKATCACCGATCC - 3'	21
P.s.PD6	5' – CTGCGCCSACCAGGTAGAG - 3'	22
P.sGGPPS1	5' - CTYGACGAYATGCCCTGCATGGAC – 3' (MD92)	23
P.s.GGPPS2	5' - GTCGATTTWCCSGCGTCCTKATTG - 3' (MD93)	24

PCR was performed in a Gradient Thermocycler, and was started by incubating at 96°C for 5 minutes, followed by 40 cycles of denaturation at 96°C for 30 seconds, annealing at 40°C/45°C/50°C/55°C/or 60°C for 105 seconds, and extension at 72°C for 90 seconds, followed by incubation at 72°C for 10 mins. The concentration of $MgCl_2$ in the PCR reactions also was varied and ranged from a final concentration of 1.5 mM to 6 mM. Table 4 provides the predicted size of the PCR products with various primer combinations.

TABLE 4
Expected sizes of PCR Products

Primer Combination	PCR product length (bp)	Product Observed
BCHy1/BCHy2	230	Yes
PS1/PS1	410	Yes
LBC1/LBC3	320	Yes
LBC1/LBC4	460	Yes
PD1/PD2	420	No
PD1/PD4	1260	No
LBC2/LBC3	240	No
PD3/PD4	410	Yes
LBC2/LBC4	380	Yes
PD5/PD6	1200	Yes
PS1/PS2	410	Yes
BCHy1/BCHy2	230	Yes
PsGGPPS1/PsGGPPS2	470	Yes
LBCDown1/PDUp1	470	Yes
PDDown1/PSUp1	300	Yes
BCHyDown1/PSDown1	700	Yes
LBCUp1/GGPPSdn1	1600	Yes

PCR reactions were electrophoresed through agarose gels to estimate sizes of PCR products and DNA was extracted from the gel using a Qiagen gel extraction kit. The purified PCR products were submitted to the Advanced Genetic Analysis Center (AGAC) at the University of Minnesota for sequencing. The obtained DNA sequences were subjected to BLAST analysis to determine if the sequences were homologous to *crt* genes from other bacteria. Sequence analysis of the 1.2-kb DNA fragment indicated that there was homology to phytoene desaturase (*crtI*) genes from *E. herbicola* and *E. uredovora*, while the 0.47 kB product had homology with the *crtE* genes from *E. herbicola* and *E. uredovora.*

Based on the DNA sequence information generated using the degenerate primers and amplified regions of the carotenoid genes from *P. stewartii*, primers specific for the *P. stewartii crt* genes were designed and are shown in Table 5. These specific primers were used to obtain information upstream and downstream of the DNA regions amplified

with the degenerate primers. This rationale was used to extend and obtain DNA sequence information about the *P. stewartii crt* genes.

TABLE 5
***P. stewartii* primers**

Primer	Sequence	SEQ ID NO
PsOp.crtE	5'-GGCCGAATTCCAACGATGCTCTGGCAGTTA-3'	25
PSOp.crtZ(-)	5'-GGCCAGATCTACTTCAGGCGACGCTGAGAG-3'	26
PsOp.crtZ(+)	5'-GGCCAGATCTTACGCGCGGGTAAAGCCAAT-3'	27
PsOp.crtZ(2+)	5'-GGCCTCTAGAATTACCGCGTGGTTCTGAAG-3'	28
PsOp.crtZ(2-)	5'-GGCCTCTAGATCTGTACGCGCCACCGTTAT-3'	29

After unsuccessful attempts at completing the sequence *crt* gene cluster sequence from *P. stewartii* using PCR, the Universal Genome Walker kit from Clontech was used to obtain the complete the sequence of the *P. stewartii crtE* and *crtZ* genes. This kit uses a PCR based approach. The following primer pairs were synthesized and used for the genome walking experiments: GWcrtE2, 5' - CATCGGTAAGATCGTCAAGCAACTGAA - 3' (SEQ ID NO:30) and GWcrtE1, 5' - GATTTACCTGCATCCTGATTGATGTCT - 3' (SEQ ID NO:31); and GWcrtZ1, 5' - ATGTATAACCGTTTCAGGTAGCCTTTG - 3' (SEQ ID NO:32) and GWcrtZ2, 5' - AATACAGTAAACCATAAGCGGTCATGC - 3' (SEQ ID NO:33). The sequences of the *crt* genes and encoded proteins from *P. stewartii* were compared to the sequence of the crt genes and proteins from *E. herbicola* and *E. uredovora* using BLAST under default parameters. See, SEQ ID NOS 1-12 for the nucleotide and amino acid sequences of the *P. stewartii crt* genes. The results of the alignment are provided in Table 6.

TABLE 6
Comparison of crt genes and proteins from *P. stewartii* to *E. herbicola* and *E. uredovora*

	Comparison of nucleotide sequence of *P. stewartii* to		Comparison of protein sequence of *P. stewartii* to	
Gene	*E. herbicola*	*E. uredovora*	*E. herbicola*	*E. uredovora*
crtE	59%	80%	81%	83%
crtX	56%	75%	75%	74%
crtY	58%	77%	83%	82%

	Comparison of nucleotide sequence of *P. stewartii* to		Comparison of protein sequence of *P. stewartii* to	
Gene	*E. herbicola*	*E. uredovora*	*E. herbicola*	*E. uredovora*
crtI	69%	81%	89%	89%
crtB	63%	81%	88%	88%
crtZ	65%	84%	65%	88%

Example 2 - Cloning of a β-carotene C4 Oxygenase from *Brevundimonas aurantiaca*: Degenerate PCR primers for *crtW* were designed based on *crtW* genes from *Bradyrhizobium*, *Alcaligenes*, *Agrobacterium aurantiacum*, and *Paracoccus marcusii*. The primers had the following sequences: (*crtW*(181P.m.) - 5'TTCATCATCGCGCATGAC3' (SEQ ID NO:34) and *crtW*(668P.m.)- 5'AGRTGRTGYTCGTGRTGA (SEQ ID NO:35), and were synthesized by Integrated DNA Technologies Inc. (Coralville, IA). PCR was performed in a mastercycler gradient machine (Eppendorf) with genomic DNA from *B. aurantiaca* (ATCC Accession No. 15266). Reaction conditions included five minutes at 96°C, followed by 30 cycles of denaturation at 94°C for 30 sec., annealing at 50°C for 2 min., and extension at 72°C for 2 min 30 sec, and a final 72°C incubation for 10 min. An approximately 500-bp PCR product was obtained and cloned into the vector pCR-BluntII-TOPO (Invitrogen Corp. Carlsbad, CA).

Independent clones were sequenced using the universal M13 forward and reverse primers. DNA sequencing was carried out at AGAC, University of Minnesota, St. Paul, MN. Partial nucleotide sequence of the *crtW* gene was obtained. Alignment of the partial sequence with known *crtW* genes indicated that the sequences aligned toward the N-terminus and C-terminus, respectively, of the *crtW* genes from *Bradyrhizobium*, *Alcaligenes*, *Agrobacterium aurantiacum*, and *Paracoccus marcusii*. The Universal Genome Walker kit from Clontech was used to obtain the complete the sequence of the *B. aurantiaca crtW* gene. Primers were synthesized based on the partial sequence and used for the genome walking experiments.

Upon obtaining sequence from the ends of the gene, the following oligonucleotide primers were synthesized and used to amplify the complete *crtW* gene from genomic DNA: 5'-GCGGCATAGGCTAGATTGAAG-3' (primer 1, Tm = 72°C, SEQ ID NO:36) and 5'-GCGAGTTCCTTCTCACCTAT-3' (primer 2, Tm = 67°C, SEQ ID NO:37). *B.*

aurantiaca (ATCC 15266) genomic DNA was prepared with the Qiagen genomic-tip 500G kit (Valencia, CA; Catalog # 10262) following the manufacturers protocol. Briefly, 30 ml of *B. aurantiaca* culture were grown overnight at 30°C in ATCC medium 36 (Caulobacter medium; 2g/l peptone, 1 g/l yeast extract, 0.2 g/l MgSO4.7H20). Cultures were harvested by centrifugation (15,000 x g; 10 minutes) and genomic DNA purified following the manufacturer's recommended protocol (Qiagen Genomic DNA Handbook for Blood, Cultured Cells, Tissue, Mouse Tails, Yeast, Bacteria (Gram- & some Gram+). The Expand DNA polymerase system (Roche Molecular Biochemicals, Indianapolis, IN; catalog # 1732641) was used in a reaction that included 2 µl of *B. aurantiaca* genomic DNA (50 ng/µl), 1 µl of primer 1 (100 pmol/µl), 1 µl of primer 2 (100 pmol/µl), 5 µl of 10x PCR buffer, 1 µl of Expand DNA polymerase (3.5 U/µl), 2.5 µl of dimethyl sulfoxide (DMSO), 2 µl of dNTP's (10 nmol/µl each), and 35.5 µl of dd H_2O. Reaction conditions included five minutes at 96°C, followed by 30 cycles of denaturation at 94°C for 30 sec., annealing at 50°C for 2 min., and extension at 72°C for 2 min 30 sec, and a final 72°C incubation for 10 min.

PCR products were electrophoresed through a 0.8% agarose gel and the ~0.85 kB band was excised from the gel and purified using the Qiagen QIAquick Gel Extraction Kit (catalog #28704) following the manufacturer's recommended protocol (QIAquick Spin Handbook). Gel-purified PCR product was cloned into the blunt-end cloning site of pCR-Blunt II-TOPO (Clontech; Palo Alto, CA) to generate pTOPOcrtW. Ligation mixtures were electroporated (25 µF, 200 Ohms, 12.5 KV/cm) into *E. coli* DH10B electromax cells (Gibco BRL; Gaithersburg, MD; catalog #18290-015). Transformants were allowed to recover 60 minutes at 37°C with shaking in 1 ml of SOC medium. Cells were plated on LB agar + 50 µg/ml kanamycin and allowed to grow overnight at 37°C. Transformant colonies were inoculated into 1 ml LB broth + 50 µg/ml kanamycin and allowed to grow overnight at 37°C with shaking. Minipreps were prepared using the QIAprep Spin Miniprep Kit (50) (catalog #27104) following the manufacturer's protocol and the presence of pTOPOcrtW was screened for by restriction analysis with *Eco*RI. *Eco*RI digests of pTOPOcrtW yielded products of ~0.85 Kbp and 3.5 Kbp.

The *crtW* gene was sequenced by AGAC, University of Minnesota, St. Paul, MN. The nucleotide sequence of the *crtW* gene from *B. aurantiaca* is provided in SEQ ID NO:38, and the protein encoded by the *crtW* gene is provided in SEQ ID NO:39.

Example 3 - Transformation of pTOPOcrtW into *Pantoea stewartii* and production of astaxanthin and adonixanthin in *P.stewartii*::pTOPOcrtW: The following protocol describes expression of *crtW* in the zeaxanthin producing host *P. stewartii*. This yields a transformed host that is capable of producing astaxanthin (i.e., 3,3'-dihydroxy-β,β-carotene-4,4'-dione) and adonixanthin (3,3'-dihydroxy-β,β-carotene-4-one). Electrocompetent *P. stewartii* (ATCC 8200) cells were prepared by culturing 50 ml of a 5% inoculum of *P. stewartii* cells in LB at 30°C -with agitation (250 rpm) until an OD_{590} of 0.5-1.0 was reached. The bacteria were washed in 50 ml of 10mM HEPES (pH 7.0) and centrifuged for 10 minutes at 10,000xg. The wash was repeated with 25 ml of 10mM HEPES (pH 7.0) followed by the same centrifugation protocol. The cells then were washed once in 25 ml of 10% glycerol. Following centrifugation, the cells were resuspended in 500 µl of 10% glycerol. Forty µl aliquots were frozen and kept at –80°C until use.

Plasmid TOPOcrtW was electroporated into electrocompetent *P. stewartii* cells (25 µF, 25 KV/cm, 200 Ohms) and plated onto LB agar plates containing 50 µg/ml kanamycin. As a negative control, pCR-Blunt II-TOPO self-ligated parental vector also was electroporated into *P. stewartii* and plated onto LB agar plates containing 50 µg/ml kanamycin. Individual colonies of *P. stewartii*::pTOPOcrtW were screened by visual inspection for a phenotypic change from bright yellow pigmentation (production of zeaxanthin) to a reddish-orange pigmentation (production of astaxanthin) and chosen for further pigment analysis. No phenotypic change was noted for individual colonies of *P. stewartii*:: pCR-Blunt II-TOPO, so clones were randomly chosen for pigment analysis.

Production of astaxanthin was confirmed by HPLC/MS. Carotenoids were extracted from cells harvested from 5 day old cultures of *P. stewartii*::pTOPOcrtW or *P. stewartii*:: pCR-Blunt II-TOPO (25 ml) grown in LB with 50 µg/ml kanamycin by resuspending the washed cell pellet in 5 ml of acetone. Glass beads were added and the mixture was incubated for 60 minutes at room temperature in the dark with occasional

vortexing. The cells were separated from the acetone extract by centrifugation at 15,000 x g for 10 minutes. The acetone supernatant then was analyzed by HPLC/MS.

A Waters 2790 LC system was used with two reverse-phase C30 specialty columns designed for carotenoid separation (YMCa Carotenoid S3m; 2.0 X 150 mm, 3 mm particle size; Waters Corporation, PN CT99S031502WT)), in tandem. The columns were run at room temperature. A gradient of Mobile Phase A (0.1% acetic acid) and Mobile Phase B (90% acetone) was used to separate zeaxanthin and astaxanthin according to the following gradient timetable: 0 min (10%A, 90%B), 10 min (100%B), 12 min (10%A, 90%B), 15 min (10%A, 90%B). Flow rate was 0.3 ml/min. Samples were stored at 20°C in an autosampler and a volume of 25 µL was injected. A Waters 996 Photodiode array detector, 350-550 nm, was used to detect zeaxanthin and astaxanthin. Under these chromatography conditions astaxanthin eluted at approximately 5.42-5.51 min and zeaxanthin eluted at approximately 6.22-6.4 min.

Carotenoid standards were used to identify the peaks. Astaxanthin was obtained from Sigma Chemical Co. (St. Louis, MO) and zeaxanthin was obtained from Extrasynthese (France). UV-Vis absorbtion spectra were used as diagnostic features for the carotenoids as were the molecular ion and fragmentation patterns generated using mass spectrometry. A positive-ion atmospheric pressure chemical ionization mass spectrometer was used; scan range, 400-800 m/z with a quadripole ion trap. A representative HPLC chromatogram is shown in FIG 3, which confirms production of astaxanthin in *P. stewartii* transformed with the *B. aurantiaca crtW* gene.

Example 4 - Simultaneous Production of CoQ-10 and (3S, 3'S) Astaxanthin in a Microorganism: Although *Phaffia rhodozyma* is not capable of producing the 3S, 3'S isoform of astaxanthin, it is known to produce Coenzyme Q-10. This compound has been found to have particularly high value as a nutraceutical. The current invention is of particular value since *R. sphaeroides* is known to produce Coenzyme Q-10 and has been transformed with genes that, while novel, are nevertheless homologous to native genes in the MABP. Consequently, the described organism can be expected to simultaneously produce both Coenzyme Q-10 and (3S, 3'S)-ATX. This is the first described production of the production of both (3S, 3'S)-ATX and Coenzyme Q-10 in a single microbial host.

The identification of (3S, 3'S)-ATX can be accomplished as described by Maoka, T., et al. J. Chromatogr. 318:122-124 (1985). Briefly, this consists of extraction of the carotenoid pigments by contacting the biomass with a suitable organic solvent such as actetone or dichloromethane. The carotenoid extract is then dried under a stream of liquid nitrogen and resuspended in a solvent of n-hexane-dichloromethane-ethanol (48:16:0.6). The extract is applied to a Sumipax OA-2000 (particle size 10uM) 250 x 4 mm I.D. (Sumitomo Chemicals, Osaka, Japan) chiral resolution HPLC column at a flow rate of 0.8 ml/min. Generally, the order of elution is expected to be (3R, 3'R)-ATX followed by (3R, 3'S; 3S, 3'R)-ATX followed by (3S, 3'S)-ATX. A similar separation is described in Maoka, T., et al. Comp. Biochem. Physiol. 83B:121-124 (1986). Briefly, this consists of isolation of the carotenoid, derivitization to the dibenzoate form with benzoyl chloride and separation of the enantiomers using a Sumipax OA-2000 chiral resolution HPLC column.

Example 5 – Transformation of the multifunctional GGPP synthase from *Archeoglobus fulgidus* into *Rhodobacter* strain ppsr- with the *crtY* and *crtI* genes from *Pantoea stewartii* inserted into the chromosome: The following protocol describes the generation of a β-carotene producing strain of *R. sphaeroides* (ATCC 35053), a facultative photoheterotroph, in which the *ppsr* gene was deleted by using the in-frame deletion procedure of Higuchi, R., et al, Nucleic Acid Res. 16: 7351-7367 to generate strain ΔREG. Table 7 describes the strains and plasmids used in this example. PpsR is a transcription factor that is involved in the repression of photosysem gene expression under aerobic growth conditions. The region of the chromosome that included the native *tspO*, *crtC*, *crtD*, *crtE* and *crtF* genes of ΔREG were replaced by the lycopene β cyclase (*crtY*) and phytoene desaturase (*crtI*) genes from *P. stewartii* using the procedure of Oh and Kaplan, Biochemistry 38:2688-2696 (1999); and Lenz, et al., J. Bacteriology 176:4385-4393 (1994), to generate the strain ΔREG(Δ5:YI). Briefly, the *crtY* and *crt I* genes were cloned into pLO1, a suicide vector for *R. sphaeroides* containing the Kanamycin resistance gene and the *Bacillus subtilis sacB* gene encoding sensitivity to sucrose. DNA fragments flanking the *crtYI* genes and identical in sequence to ~500 bp internal fragments of the *R. sphaeroides tspO* and *crtF* genes were then cloned

into pLO1. These flanking DNA regions correspond to the desired region for insertion of the *crtYI* genes. Insertion of the *crtYI* genes in ΔREG was confirmed using PCR analyses and appropriate PCR primers specific to the *crtYI* genes as well as flanking regions of the *R.sphaeroides* genome. The *crtYI* (*P. stewartii*) insertion and *tspO, crtC, crtD, crtE* and *crtF* (*R. sphaeroides*) deletion resulted in the lack of native carotenoid production and a change in the pigmentation from red to green, confirming the insertion event.

TABLE 7

Description of *Rhodobacter* Strains and Plasmids

Strain	Description	Major Carotenoid Produced	Comments
ΔREG	ATCC 35053; *ppsR* regulatory mutant	Sphaeroidenone (Native Carotenoid)	Regulatory mutant
ΔREG(Δ5:YI)	*CrtY* and *crtI* genes of *P. stewartii* replaced 5 host genes (*tspO*, *crtC*, *crtD*, *crtE* and *crtF*) on chromosome	None	β-carotene biosynthetic genes placed in chromosome. No carotenoid production because of *crtE* deletion
ΔREG(Δ5:YI)::pPctrl	Control vector introduced into ΔREG(Δ5:YI) host	None	Control vector contains *rrnB* promoter but no biosynthetic genes
ΔREG(Δ5:YI)::pPgps	*gps* gene of *A. fulgidus* inserted into pPctrl control vector and introduced into ΔREG(Δ5:YI) host	β-Carotene	*gps* gene on plasmid complements *crtE* deletion. Complete pathway for β-carotene production

Strain	Description	Major Carotenoid Produced	Comments
ΔREG(Δ5:YI) (ΔA:gps)	*gps* gene of *A. fulgidus* replaced *crtA* host gene on chromosome of ΔREG(Δ5:YI) host	β-Carotene	*gps* gene inserted into genome complements *crtE* deletion. Complete pathway for β-carotene production
ΔREG(Δ5:YI) (ΔA:gps) ::pPWZ	*crtW* and *crtZ* genes inserted into pPctrl control vector and introduced into ΔREG(Δ5:YI) (ΔA:gps) host	Astaxanthin	*crtW* and *crtZ* genes convert β-carotene into astaxanthin
ΔREG(Δ5:YI) (ΔA:gps) ::pPgpsWZ	*gps*, *crtW* and *crtZ* genes inserted into pPctrl control vector and introduced into ΔREG(Δ5:YI) (ΔA:gps) host	Astaxanthin	Additional copies of *A. fulgidus gps* gene on plasmid increases production of astaxanthin

Plasmids	Genetic elements inserted
PBBR1MCS2	None
PPctrl	*rrnB* promoter
PPgps	*rrnB* promoter, *A. fulgidus gps*
PPWZ	*rrnB* promoter, *P. stewartii crtZ*, *B. aurantiacum crtW*
PPgpsWZ	*rrnB* promoter, *A. fulgidus gps* *P. stewartii crtZ*, *B. aurantiacum crtW*

The pPctrl vector was constructed by inserting a copy of the *R. sphaeroides rrnB* promoter (GenBank Accession # X53854; rrnBP) into the vector pBBR1MCS2 (GenBank Accession # U23751). The *rrnB* promoter was isolated from the vector pTEX24 (S. Kaplan) by a *Bam*HI restriction enzyme digest, which released the promoter as a 363 bp fragment. This fragment was gel purified from a 2% Tris-acetate-EDTA (TAE) agarose gel. To prepare the pBBR1MCS2 vector for ligation, it also was digested with *Bam*HI

and the enzyme heat inactivated at 80°C for 20 minutes. The digested vector was dephosphorylated with shrimp alkaline phosphatase (Roche Molecular Biochemicals, Indianapolis, IN), and gel purified from a 1% TAE-agarose gel. The prepared vector and the *rrnB* fragment were ligated using T4 DNA ligase at 16°C for 16 hours to generate the plasmid pPctrl. One μL of ligation reaction was used to electroporate 40 μL of *E. coli* ElectromaxTM DH10BTM cells (Life Technologies, Inc., Rockville, MD).

Electroporated cells were plated on LB media containing 25 μg/mL of kanamycin (LBK). pPctrl DNA was isolated from cultures of single colonies and was digested with *Hind* III to confirm the presence of a single insertion of the *rrnB* promoter. The sequence of pPctrl also was confirmed by DNA sequencing.

The multifunctional GGPP synthase (*gps*) gene from *A. fulgidus* (GenBank Accession No. AF120272) was cloned into the multiple cloning site of pPctrl to generate the construct pPgps.

Electrocompetent ΔREG(Δ5:YI) cells were prepared as follows: 5 ml cultures were inoculated using Sistrom's media supplemented with trace elements, vitamins (O'Gara, et al., J. Bacteriol. 180:4044-4050 (1988); Cohen-Bazire, et al. J. Cell. Comp. Physiol. 49:25-68 (1957)) and 0.4% glucose as a carbon source, and grown overnight at 30°C with shaking. This culture was diluted 1/100 in 300 mL of the same media and grown to an OD_{660} of 0.5-0.8. The cells were chilled on ice for 10 minutes and then centrifuged for 6 minutes at 7,500 g. The supernatant was discarded and the cell pellet was resuspended in ice-cold 10% glycerol at half of the original volume. The cells were pelleted by centrifugation for 6 minutes at 7,500 g. The supernatant was again discarded and cells were resuspended in ice cold 10% glycerol at one quarter of the original volume. The last centrifugation and resuspension steps were repeated, followed by centrifugation for 6 minutes at 7,500 g. The supernatant was decanted and the cells resuspended in the small volume of glycerol that did not drain out. Additional ice-cold 10% glycerol was added to resuspend the cells if necessary. Forty μL of the resuspended cells was used in a test electroporation (see below) to determine if the cells needed to be concentrated by centrifugation or diluted with 10% ice-cold glycerol. Time constants of 8.5-9.0 resulted in good transformation efficiencies. Once an acceptable time constant was achieved, cells

were aliquoted into cold microfuge tubes and stored at -80°C. All water used for media and glycerol was 18 Mohm or higher.

Electroporation of ΔREG(Δ5:YI) was carried out as follows. One μL of pPgps or pPctrl vector DNA was gently mixed into 40 μL of ΔREG(Δ5:YI) electrocompetent cells, which then were transferred to an electroporation cuvette with a 0.2 cM electrode gap. Electroporations were conducted using a Biorad Gene Pulser II (Biorad, Hercules, CA) with settings at 2.5 kV of potential, 400 ohms of resistance, and 25 μF of capacitance. Cells were recovered in 400 μL SOC media at 30°C for 6-16 hours. The cells were then plated, 200 μL per plate, on LB medium containing 50 μg/ml kanamycin and incubated at 30°C for 5-6 days.

After incubation, greenish colonies were observed on plates of ΔREG(Δ5:YI) transformed with pPctrl plasmid DNA. The colonies that appeared on plates of ΔREG(Δ5:YI) transformed with pPgps plasmid DNA appeared yellow. The yellow pigmentation was indicative of β-carotene production in ΔREG(Δ5:YI) expressing the *A. fulgidus gps* gene from pPgps.

Single yellow colonies were grown up in Sistrom's liquid media supplemented with vitamins, trace elements and 0.4% glucose as well as 50 μg/ml kanamycin, at 30°C with shaking for 24-48 hours. Carotenoids were extracted and subjected to LCMS analysis as described above. Under the chromatography conditions used, β-carotene eluted at approximately 13.87-14.2 min. β-carotene standard (Sigma chemical, St. Louis, MO) was used to identify the peaks. The UV-Vis absorption spectra and the retention time using HPLC were used as diagnostic features for β-carotene identification in ΔREG(Δ5:YI) transformed with pPgps DNA, as well as the molecular ion and fragmentation patterns generated during mass spectrometry. Thus, the production of β-carotene was confirmed in ΔREG(Δ5:YI) expressing the *A. fulgidus gps* gene from pPgps.

Example 6 – Transformation of the β-carotene C-4 ketolase (*crtW*) gene from *Brevumdimonas aurantiacum* and β-carotene hydroxylase (*crtZ*) from *P. stewartii* into the ΔREG(Δ5:Y1) strain of *Rhodobacter* with the *gps* gene from *Archeoglobus fulgidus* inserted into the chromosome: The following protocol describes the

generation of an astaxanthin producing strain of *R. sphaeroides* using ΔREG(Δ5:YI), described above. See also Table 7 for further description of the strains and plasmids that were used in this example. Using the gene insertion method described by Higuchi, R., et al, Nucleic Acid Res. 16: 7351-7367, the *crtA* gene of ΔREG(Δ5:YI) was replaced by the *gps* gene from *A. fulgidus* to generate the strain ΔREG(Δ5:YI)(ΔA:gps). Electrocompetent cells ΔREG(Δ5:YI)(ΔA:gps) were generated as described above.

The construct pPgpsWZ was produced by cloning the *crtW* gene from *B. aurantiacum*, the *crtZ* gene from *P.stewartii*, and the *gps* gene from *A fulgidus* into the pPctrl plasmid using appropriate restriction enzymes. The construct pPWZ was produced by cloning the *crtW* gene from *B. aurantiacum* and the *crtZ* gene from *P.stewartii* into the pPctrl plasmid using appropriate restriction enzymes.

The pPWZ or pPgpsWZ constructs were electroporated into electrocompetent ΔREG(Δ5:YI)(ΔA:gps) as described earlier to generate ΔREG(Δ5:YI)(ΔA:gps)::pPWZ or ΔREG(Δ5:YI)(ΔA:gps)::pPgpsWZ, respectively. Transformation mixtures were plated out onto LB plates containing 50 μg/ml kanamycin. PCR analyses using PCR primers specific for *crtZ* were used to confirm the presence of the pPWZ or pPgpsWZ plasmids in ΔREG(Δ5:YI)(ΔA:gps).

Single colonies of ΔREG(Δ5:YI)(ΔA:gps)::pPWZ or ΔREG(Δ5:YI)(ΔA:gps)::pPgpsWZ were grown up in media supplemented with 50 μg/ml kanamycin as described earlier. Cell pellets were washed with distilled water and then carotenoids were extracted using acetone:methanol (7:2) at 30°C for 30 mins with shaking at 225 rpm. Carotenoid analysis was performed using LCMS analysis described above. The UV-Vis absorption spectra and the retention time using HPLC were used as diagnostic features for astaxanthin identification in ΔREG(Δ5:YI)(ΔA:gps)::pPWZ and ΔREG(Δ5:YI)(ΔA:gps)::pPgpsWZ, as well as the molecular ion and fragmentation patterns generated during mass spectrometry. The production of astaxanthin was confirmed in both ΔREG(Δ5:YI)(ΔA:gps)::pPWZ and ΔREG(Δ5:YI)(ΔA:gps)::pPgpsWZ. Increased astaxanthin production was observed in ΔREG(Δ5:YI)(ΔA:gps)::pPgpsWZ.

Example 7: Cloning and sequencing of a novel multifunctional Geranylgeranyl pyrophosphate synthase gene (*gps*) from *Sulfolobus shibatae*:

Degenerate primer sequences MFGGPP1 (5'CCAYGAYGAYATWATGGA3', SEQ ID NO:40) and MFGGPP2 (5'YTTYTTVCCYTYCCTAAT3', SEQ ID NO:41) were designed based on conserved sequences in *gps* gene sequences from *Sulfolobus solfotaricus* and *Sulfolobus acidocaldarius* and synthesized by Integrated DNA Technologies (Coralville, IA). PCR was performed in a mastercycler gradient machine (Eppendorf) with genomic DNA from *S. shibatae* (ATCC Accession No. 51178, lot # 1162977). Reaction conditions included five minutes at 96°C, followed by 30 cycles of denaturation at 94°C for 30 sec., annealing at 50 + 10°C for 60 sec., and extension at 72°C for 90 sec., and a final 72°C incubation for 10 min. An approximately 500-bp PCR product was obtained and cloned into the vector pC-BuntII-TOPO (Invitrogen Corp. Carlsbad, CA).

Independent clones were sequenced using the universal M13 forward and reverse primers. DNA sequencing was carried out at the AGAC, University of Minnesota, St. Paul, MN. DNA sequence analysis of this PCR product indicated similarity to the *gps* genes from *S. sulfotaricus* and *S. acidocaldarius*. The Universal Genome Walker kit (Clontech) was used to obtain more of the *gps* gene sequence flanking the original PCR product from *S. shibatae*. Primers were synthesized based on the partial sequence and used for genome walking experiments.

The following strategy was used to completely sequence the *S. shibatae gps* gene. The ERWCRTS homolog was observed upstream of the *S. sulfotaricus gps* gene. The UDP-A-acetylglucosamine—Dolichyl-phosphate-N-acetylglucosamine phosphotransferase gene was present downstream of the gps gene in both *S. sulfotaricus* and *S. acidocaldarius*. Primers were designed based on the sequence of the two genes SsDolidn (5'ACAGCGTTGGACACTCAG 3', SEQ ID NO:42) and SsERCRTup (5' GCGTCGATAATGGAAGTGAG 3', SEQ ID NO:43) of the *gps* gene. An approximately 2 kb PCR product was amplified using the SsDolidn and SsERCRTup primers and genomic DNA from *S. shibatae*. This PCR product was cloned into the vector pC-BuntII-TOPO as described above and sequenced using the universal M13 forward and reverse primers. The nucleotide sequence of the *gps* gene from *S. shibatae* is presented in SEQ

ID NO: 44, and the amino acid sequence of the protein encoded by the *gps* gene is presented in SEQ ID NO:45.

OTHER EMBODIMENTS

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

WHAT IS CLAIMED IS:

1. An isolated nucleic acid having at least 76% sequence identity to the nucleotide sequence of SEQ ID NO:1 or to a fragment of SEQ ID NO:1 at least 33 contiguous nucleotides in length.

2. The isolated nucleic acid of claim 1, said nucleic acid having at least 80% sequence identity to the nucleotide sequence of SEQ ID NO:1.

3. The isolated nucleic acid of claim 1, said nucleic acid having at least 85% sequence identity to the nucleotide sequence of SEQ ID NO:1.

4. The isolated nucleic acid of claim 1, said nucleic acid having at least 90% sequence identity to the nucleotide sequence of SEQ ID NO:1.

5. The isolated nucleic acid of claim 1, said nucleic acid having at least 95% sequence identity to the nucleotide sequence of SEQ ID NO:1.

6. An expression vector comprising the nucleic acid of claim 1 operably linked to an expression control element.

7. An isolated nucleic acid encoding a zeaxanthin glucosyl transferase polypeptide at least 75% identical to the amino acid sequence of SEQ ID NO:2.

8. An isolated nucleic acid having at least 78% sequence identity to the nucleotide sequence of SEQ ID NO:3 or to a fragment of SEQ ID NO:3 at least 32 contiguous nucleotides in length.

9. The isolated nucleic acid of claim 8, said nucleic acid having at least 80% sequence identity to the nucleotide sequence of SEQ ID NO:3.

10. The isolated nucleic acid of claim 8, said nucleic acid having at least 85% sequence identity to the nucleotide sequence of SEQ ID NO:3.

11. The isolated nucleic acid of claim 8, said nucleic acid having at least 90% sequence identity to the nucleotide sequence of SEQ ID NO:3.

12. The isolated nucleic acid of claim 8, said nucleic acid having at least 95% sequence identity to the nucleotide sequence of SEQ ID NO:3.

13. An expression vector comprising the nucleic acid of claim 8 operably linked to an expression control element.

14. An isolated nucleic acid encoding a lycopene β-cyclase polypeptide at least 83% identical to the amino acid sequence of SEQ ID NO:4.

15. An isolated nucleic acid having at least 81% sequence identity to the nucleotide sequence of SEQ ID NO:5 or to a fragment of SEQ ID NO:5 at least 60 contiguous nucleotides in length.

16. The isolated nucleic acid of claim 15, said nucleic acid having at least 85% sequence identity to the nucleotide sequence of SEQ ID NO:5.

17. The isolated nucleic acid of claim 15, said nucleic acid having at least 90% sequence identity to the nucleotide sequence of SEQ ID NO:5.

18. The isolated nucleic acid of claim 15, said nucleic acid having at least 95% sequence identity to the nucleotide sequence of SEQ ID NO:5.

19. An expression vector comprising the nucleic acid of claim 15 operably linked to an expression control element.

20. An isolated nucleic acid encoding a geranylgeranyl pyrophosphate synthase polypeptide at least 85% identical to the amino acid sequence of SEQ ID NO:6.

21. An isolated nucleic acid having at least 82% sequence identity to the nucleotide sequence of SEQ ID NO:7 or to a fragment of SEQ ID NO:7 at least 30 contiguous nucleotides in length.

22. The isolated nucleic acid of claim 21, said nucleic acid having at least 85% sequence identity to the nucleotide sequence of SEQ ID NO:7.

23. The isolated nucleic acid of claim 21, said nucleic acid having at least 90% sequence identity to the nucleotide sequence of SEQ ID NO:7.

24. The isolated nucleic acid of claim 21, said nucleic acid having at least 95% sequence identity to the nucleotide sequence of SEQ ID NO:7.

25. An expression vector comprising the nucleic acid of claim 21 operably linked to an expression control element.

26. An isolated nucleic acid encoding a phytoene desaturase polypeptide at least 90% identical to the amino acid sequence of SEQ ID NO:8.

27. An isolated nucleic acid having at least 82% sequence identity to the nucleotide sequence of SEQ ID NO:9 or to a fragment of SEQ ID NO:9 at least 23 contiguous nucleotides in length.

28. The isolated nucleic acid of claim 27, said nucleic acid having at least 85% sequence identity to the nucleotide sequence of SEQ ID NO:9.

29. The isolated nucleic acid of claim 27, said nucleic acid having at least 90% sequence identity to the nucleotide sequence of SEQ ID NO:9.

30. The isolated nucleic acid of claim 27, said nucleic acid having at least 95% sequence identity to the nucleotide sequence of SEQ ID NO:9.

31. An expression vector comprising the nucleic acid of claim 27 operably linked to an expression control element.

32. An isolated nucleic acid encoding a phytoene synthase polypeptide at least 89% identical to the amino acid sequence of SEQ ID NO:10.

33. An isolated nucleic acid having at least 85% sequence identity to the nucleotide sequence of SEQ ID NO:11 or to a fragment of SEQ ID NO:11 at least 36 contiguous nucleotides in length.

34. The isolated nucleic acid of claim 33, said nucleic acid having at least 85% sequence identity to the nucleotide sequence of SEQ ID NO:11.

35. The isolated nucleic acid of claim 33, said nucleic acid having at least 90% sequence identity to the nucleotide sequence of SEQ ID NO:11.

36. The isolated nucleic acid of claim 33, said nucleic acid having at least 95% sequence identity to the nucleotide sequence of SEQ ID NO:11.

37. An expression vector comprising the nucleic acid of claim 33 operably linked to an expression control element.

38. An isolated nucleic acid encoding a β-carotene hydroxylase polypeptide at least 90% identical to the amino acid sequence of SEQ ID NO:12.

39. Membranous bacteria comprising at least one exogenous nucleic acid encoding phytoene desaturase, lycopene β-cyclase, β-carotene hydroxylase, and β-carotene C4

oxygenase, wherein expression of said at least one exogenous nucleic acid produces detectable amounts of astaxanthin in said membranous bacteria.

40. The membranous bacteria of claim 39, wherein the amino acid sequence of said phytoene desaturase is at least 90% identical to the amino acid sequence of SEQ ID NO:8.

41. The membranous bacteria of claim 39, wherein the amino acid sequence of said lycopene β-cyclase is at least 83% identical to the amino acid sequence of SEQ ID NO:4.

42. The membranous bacteria of claim 39, wherein the amino acid sequence of said β-carotene hydroxylase is at least 90% identical to the amino acid sequence of SEQ ID NO:12.

43. The membranous bacteria of claim 39, wherein said membranous bacteria further comprises an exogenous nucleic acid encoding geranylgeranyl pyrophosphate synthase.

44. The membranous bacteria of claim 39, wherein said membranous bacteria lacks endogenous bacteriochlorophyll biosynthesis.

45. The membranous bacteria of claim 43, wherein said exogenous nucleic acid encodes a multifunctional geranylgeranyl pyrophosphate synthase.

46. The membranous bacteria of claim 45, wherein the amino acid sequence of said multifunctional geranylgeranyl pyrophosphate synthase is at least 90% identical to the amino acid sequence of SEQ ID NO:45.

47. The membranous bacteria of claim 39, wherein the amino acid sequence of said β-carotene C4 oxygenase is at least 80% identical to the amino acid sequence of SEQ ID NO:39.

48. The membranous bacteria of claim 39, wherein said membranous bacteria further comprise an exogenous nucleic acid encoding phytoene synthase.

49. The membranous bacteria of claim 48, wherein the amino acid sequence of said phytoene synthase is at least 89% identical to the amino acid sequence of SEQ ID NO:10.

50. The membranous bacteria of claim 39, wherein said membranous bacteria are a *Rhodobacter* species.

51. Membranous bacteria, said membranous bacteria comprising an exogenous nucleic acid encoding a phytoene desaturase having an amino acid sequence at least 90% identical to the amino acid sequence of SEQ ID NO:8, and wherein said membranous bacteria produces detectable amounts of lycopene.

52. The membranous bacteria of claim 51, wherein said membranous bacteria further comprise a lycopene β-cyclase, and wherein said membranous bacteria produce detectable amounts of β-carotene.

53. The membranous bacteria of claim 52, wherein said membranous bacteria further comprise a β-carotene hydroxylase, and wherein said membranous bacteria produce detectable amounts of zeaxanthin.

54. Membranous bacteria comprising at least one exogenous nucleic acid encoding phytoene desaturase, lycopene β-cyclase, and β-carotene C4 oxygenase, wherein expression of said at least one exogenous nucleic acid produces detectable amounts of canthaxanthin in said membranous bacteria.

55. A composition comprising an engineered *Rhodobacter* cell, wherein said cell produces a detectable amount of astaxanthin or canthaxanthin.

56. The composition of claim 55, wherein said engineered *Rhodobacter* cell comprises at least one exogenous nucleic acid encoding phytoene desaturase, lycopene β-cyclase, β-carotene hydroxylase, and β-carotene C4 oxygenase.

57. The composition of claim 55, wherein said composition is formulated for aquaculture.

58. The composition of claim 57, wherein said composition pigments the flesh of fish or the carapace of crustaceans after ingestion.

59. The composition of claim 55, wherein said composition is formulated for human consumption.

60. The composition of claim 55, wherein said composition is formulated as an animal feed.

61. The composition of claim 60, wherein said animal feed is formulated for consumption by chickens, turkeys, cattle, swine, or sheep.

62. A method of making a nutraceutical, said method comprising extracting carotenoids from an engineered *Rhodobacter* cell, said engineered *Rhodobacter* cell comprising at least one exogenous nucleic acid encoding phytoene desaturase, lycopene β-cyclase, β-carotene hydroxylase, and β-carotene C4 oxygenase, and wherein said *Rhodobacter* cell produces detectable amounts of astaxanthin.

63. Membranous bacteria, said membranous bacteria comprising an exogenous nucleic acid encoding a lycopene β-cyclase having an amino acid sequence at least 83% identical to the amino acid sequence of SEQ ID NO:4.

64. The membranous bacteria of claim 63, said membranous bacteria further comprising a phytoene desaturase, wherein said membranous bacteria produces detectable amounts of β-carotene.

65. The membranous bacteria of claim 64, said membranous bacteria further comprising a β-carotene hydroxylase, wherein said bacteria produces detectable amounts of zeaxanthin.

66. Membranous bacteria, said membranous bacteria comprising a β-carotene hydroxylase having an amino acid sequence at least 90% identical to the amino acid sequence of SEQ ID NO:12.

67. The membranous bacteria of claim 66, said membranous bacteria further comprising a lycopene β-cyclase, and wherein said membranous bacteria produces detectable amounts of zeaxanthin.

68. The membranous bacteria of claim 67, said membranous bacteria further comprising a phytoene desaturase, wherein said membranous bacteria produces detectable amounts of β-carotene.

69. Membranous bacteria, said bacteria lacking an endogenous nucleic acid encoding a farnesyl pyrophosphate synthase, and wherein said bacteria produce detectable amounts of carotenoids.

70. The membranous bacteria of claim 69, wherein said bacteria comprise an exogenous nucleic acid encoding a multifunctional geranylgeranyl pyrophosphate synthase.

71. The membranous bacteria of claim 70, wherein the amino acid sequence of said multifunctional geranylgeranyl pyrophosphate synthase is at least 90% identical to the amino acid sequence of SEQ ID NO:45.

72. The membranous bacteria of claim 69, wherein said membranous bacteria are a species of *Rhodobacter*.

73. An isolated nucleic acid having at least 60% sequence identity to the nucleotide sequences of SEQ ID NO:38, or to a fragment of the nucleic acid of SEQ ID NO:38 at least 15 contiguous nucleotides in length.

74. The isolated nucleic acid of claim 73, said nucleic acid having at least 80% sequence identity to the nucleotide sequences of SEQ ID NO:38, or to a fragment of the nucleic acid of SEQ ID NO:38 at least 15 contiguous nucleotides in length.

75. The isolated nucleic acid of claim 73, said nucleic acid having at least 90% sequence identity to the nucleotide sequences of SEQ ID NO:38, or to a fragment of the nucleic acid of SEQ ID NO:38 at least 15 contiguous nucleotides in length.

76. The isolated nucleic acid of claim 73, wherein said nucleic acid encodes a β-carotene C4 oxygenase.

77. Membranous bacteria comprising an exogenous nucleic acid encoding a β-carotene C4 oxygenase, said β-carotene oxygenase having an amino acid sequence at least 80% identical to the amino acid sequence of SEQ ID NO:39.

78. A host cell comprising an exogenous nucleic acid, wherein the exogenous nucleic acid comprises a nucleic acid sequence encoding one or more polypeptides that catalyze the formation of (3S, 3'S) astaxanthin, wherein the host cell produces CoQ-10 and (3S, 3'S) astaxanthin.

79. A method of making CoQ-10 and (3S, 3'S) astaxanthin at substantially the same time, the method comprising transforming a host cell with a nucleic acid, wherein the nucleic acid comprises a nucleic acid sequence that encodes one or more

polypeptides, wherein the polypeptides catalyze the formation of (3S, 3'S) astaxanthin; and culturing the host cell under conditions that allow for the production of (3S, 3'S) astaxanthin and CoQ-10.

80. The method of claim 79, additionally comprising transforming the host cell with at least one exogenous nucleic acid, the exogenous nucleic acid encoding one or more polypeptides, wherein the polypeptides catalyze the formation of CoQ-10.

81. An isolated nucleic acid having a nucleotide sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:38, and SEQ ID NO:44.

82. An isolated nucleic acid having at least 90% sequence identity to the nucleotide sequences of SEQ ID NO:44, or to a fragment of the nucleic acid of SEQ ID NO:44 at least 60 contiguous nucleotides in length.

83. A method of making geranylgeranyl pyrophosphate, said method comprising contacting isopentenyl pyrophosphate and dimethylallyl pyrophosphate with a polypeptide encoded by the isolated nucleic acid of claim 82.

84. A method of making geranylgeranyl pyrophosphate, said method comprising contacting farnesyl pyrophosphate and isopentenyl pyrophosphate with a polypeptide encoded by the isolated nucleic acid of claim 15 or the polypeptide of claim 20.

85. A method of making β-carotene, said method comprising contacting lycopene with a polypeptide encoded by the isolated nucleic acid of claim 8 or the polypeptide of claim 14.

86. A method of making lycopene, said method comprising contacting phytoene with a polypeptide encoded by the isolated nucleic acid of claim 21 or the polypeptide of claim 26.

87. A method of making phytoene, said method comprising contacting geranylgeranyl pyrophosphate with a polypeptide encoded by the isolated nucleic acid of claim 27 or the polypeptide of claim 32.

88. A method of making zeaxanthin, said method comprising contacting β-carotene with a polypeptide encoded by the isolated nucleic acid of claim 33 or the polypeptide of claim 38.

89. A method of making canthaxanthin, said method comprising contacting β-carotene with a polypeptide encoded by the isolated nucleic acid of claim 73 or a polypeptide having an amino acid sequence at least 80% identical to the amino acid sequence of SEQ ID NO:39.

90. A method of making astaxanthin, said method comprising contacting canthaxanthin with a polypeptide encoded by the isolated nucleic acid sequence of claim 33 or the polypeptide of claim 38.

91. A method of making astaxanthin, said method comprising contacting zeaxanthin with a polypeptide encoded by the isolated nucleic acid sequence of claim 73 or a polypeptide having an amino acid sequence at least 80% identical to the amino acid sequence of SEQ ID NO:39.

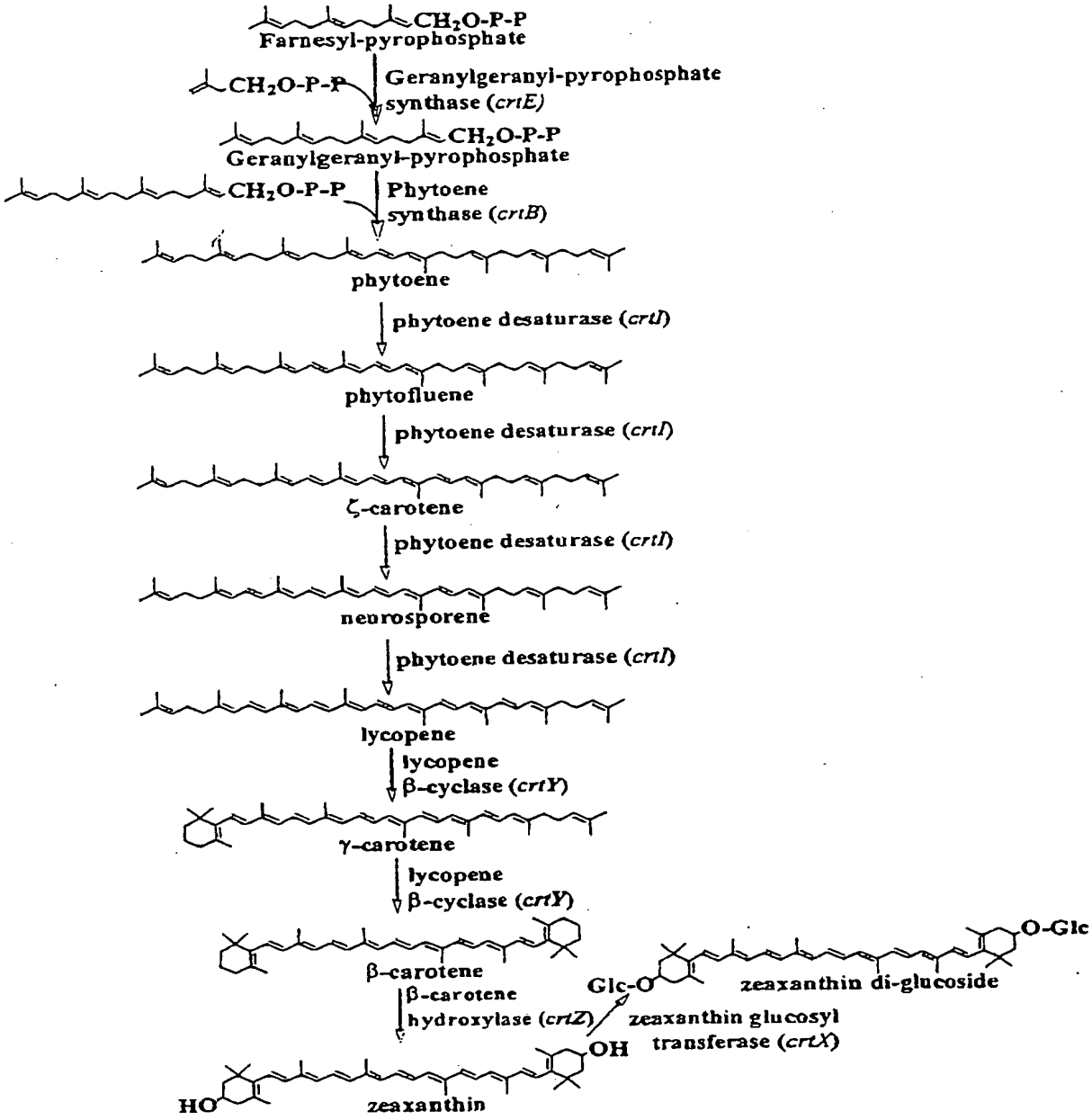

FIG. 1

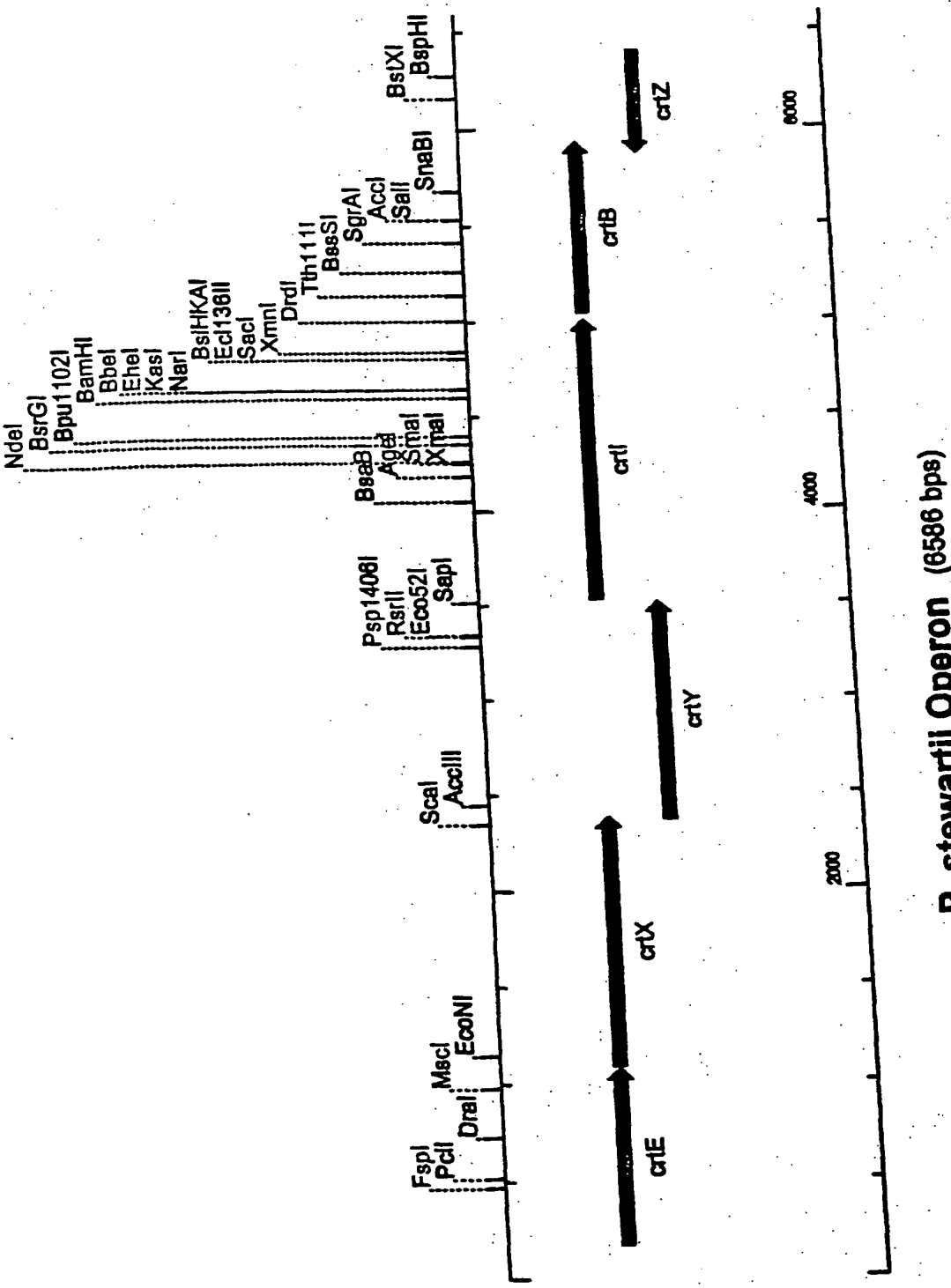

FIG. 2

HPLC Analysis

Pantoea stewartii – zeaxanthin production

P.stewartii:: *crtW* (*Brevundimonas aurantiaca*) – astaxanthin production

Zeaxanthin standard

Astaxanthin standard

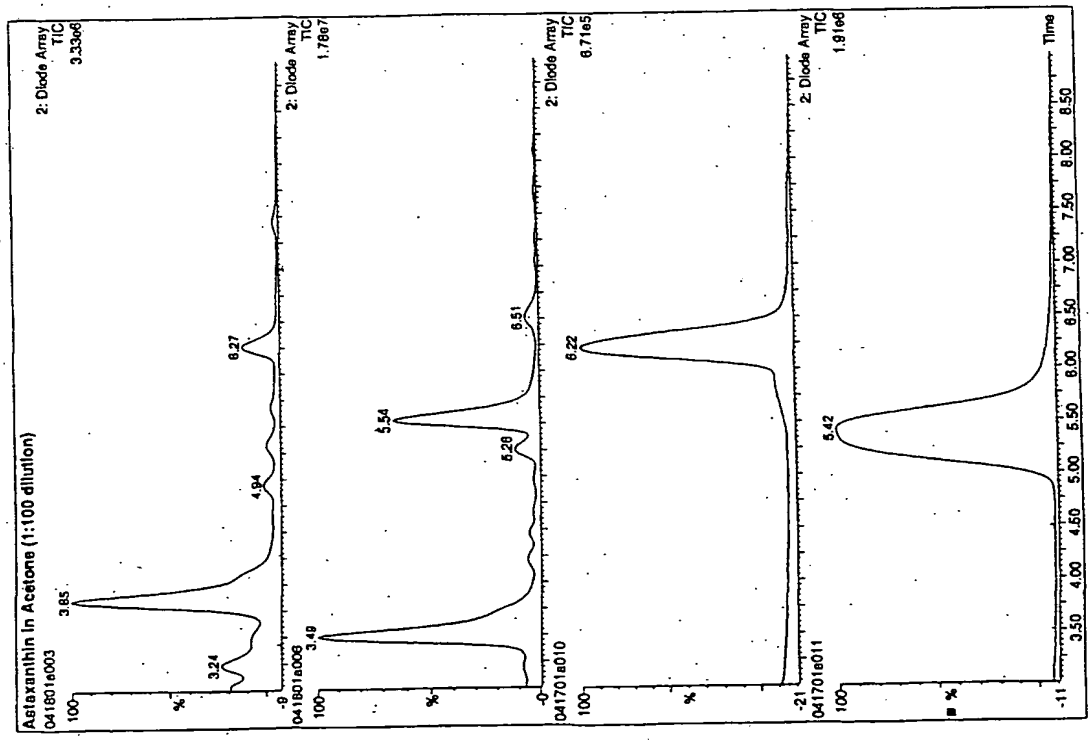

FIG. 3

SEQUENCE LISTING

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Arg Ala Val Phe Asn Glu Gly Tyr Leu Lys Leu Gly Thr Val Pro Phe
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Val Gln Leu Glu Asp Gly Arg Arg Phe Glu Thr Cys Ala Val Ala Ser
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Ser Tyr Tyr Val Leu Ala Pro Val Pro His Leu Gly Thr Ala Asn Leu
    370                 375                 380
Asp Trp Ala Val Glu Gly Pro Arg Leu Arg Asp Arg Ile Phe Asp Tyr
385                 390                 395                 400
Leu Glu Gln His Tyr Met Pro Gly Leu Arg Ser Gln Leu Val Thr His
                405                 410                 415
Arg Met Phe Thr Pro Phe Asp Phe Arg Asp Glu Leu Asn Ala Trp Gln
            420                 425                 430
Gly Ser Ala Phe Ser Val Glu Pro Ile Leu Thr Gln Ser Ala Trp Phe
        435                 440                 445
Arg Pro His Asn Arg Asp Lys His Ile Asp Asn Leu Tyr Leu Val Gly
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        35                  40                  45
Pro Ser Ser Gln Met Pro Glu Gln Arg Leu Gln Gln Leu Glu Met Lys
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Thr Arg Gln Ala Tyr Ala Gly Ser Gln Met His Glu Pro Ala Phe Ala
65                  70                  75                  80
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Ala Phe Gln Glu Val Ala Met Ala His Asp Ile Ala Pro Ala Tyr Ala
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Val Gly Leu Met Met Ala Gln Ile Met Gly Val Arg Asp Asn Ala Thr
    130                 135                 140
Leu Asp Arg Ala Cys Asp Leu Gly Leu Ala Phe Gln Leu Thr Asn Ile
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Ala Arg Asp Ile Val Asp Asp Ala Gln Val Gly Arg Cys Tyr Leu Pro
                165                 170                 175
Glu Ser Trp Leu Glu Glu Glu Gly Leu Thr Lys Ala Asn Tyr Ala Ala
            180                 185                 190
Pro Glu Asn Arg Gln Ala Leu Ser Arg Ile Ala Gly Arg Leu Val Arg
        195                 200                 205
Glu Ala Glu Pro Tyr Tyr Val Ser Ser Met Ala Gly Leu Ala Gln Leu
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Pro Leu Arg Ser Ala Trp Ala Ile Ala Thr Ala Lys Gln Val Tyr Arg
225                 230                 235                 240
Lys Ile Gly Val Lys Val Glu Gln Ala Gly Lys Gln Ala Trp Asp His
                245                 250                 255
Arg Gln Ser Thr Ser Thr Ala Glu Lys Leu Thr Leu Leu Leu Thr Ala
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gtgtcgattg ccctgattta cttcggcagt acaggaatct ggccgctcca gtggattggt     240
gcaggcatga ccgcttatgg tttactgtat tttatggtcc acgacggact ggtacaccag     300
cgctggccgt tccgctacat accgcgcaaa ggctacctga aacggttata catggcccac     360
cgtatgcatc atgctgtaag gggaaaagag ggctgcgtgt cctttggttt tctgtacgcg     420
ccaccgttat ctaaacttca ggcgacgctg agagaaaggc atgcggctag atcgggcgct     480
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            20                  25                  30
Gly Trp Gly Trp His Leu Ser His His Glu Pro Arg Lys Gly Ala Phe
        35                  40                  45
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Glu Val Asn Asp Leu Tyr Ala Val Val Phe Ala Ile Val Ser Ile Ala
50 55 60
Leu Ile Tyr Phe Gly Ser Thr Gly Ile Trp Pro Leu Gln Trp Ile Gly
65 70 75 80
Ala Gly Met Thr Ala Tyr Gly Leu Leu Tyr Phe Met Val His Asp Gly
85 90 95
Leu Val His Gln Arg Trp Pro Phe Arg Tyr Ile Pro Arg Lys Gly Tyr
100 105 110
Leu Lys Arg Leu Tyr Met Ala His Arg Met His His Ala Val Arg Gly
115 120 125
Lys Glu Gly Cys Val Ser Phe Gly Phe Leu Tyr Ala Pro Pro Leu Ser
130 135 140
Lys Leu Gln Ala Thr Leu Arg Glu Arg His Ala Ala Arg Ser Gly Ala
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Ala Arg Asp Glu Gln Asp Gly Val Asp Thr Ser Ser Ser Gly Lys
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<210> 15
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<210> 17
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<400> 21

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<400> 33 27
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<400> 34 18
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<400> 35 18
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35 40 45
Ile Ala Pro Ala Ile Val Ala Val Gln Thr Trp Leu Ser Val Gly Leu
50 55 60
Phe Ile Val Ala His Asp Ala Met Tyr Gly Ser Leu Ala Pro Gly Arg
65 70 75 80
Pro Arg Leu Asn Ala Ala Val Gly Arg Leu Thr Leu Gly Leu Tyr Ala
85 90 95
Gly Phe Arg Phe Asp Arg Leu Lys Thr Ala His His Ala His His Ala
100 105 110
Ala Pro Gly Thr Ala Asp Asp Pro Asp Phe His Ala Pro Ala Pro Arg
115 120 125
Ala Phe Leu Pro Trp Phe Leu Asn Phe Phe Arg Thr Tyr Phe Gly Trp
130 135 140
Arg Glu Met Ala Val Leu Thr Ala Leu Val Leu Ile Ala Leu Phe Gly
145 150 155 160
Leu Gly Ala Arg Pro Ala Asn Leu Leu Thr Phe Trp Ala Ala Pro Ala
165 170 175
Leu Leu Ser Ala Leu Gln Leu Phe Thr Phe Gly Thr Trp Leu Pro His
180 185 190

Arg His Thr Asp Gln Pro Phe Ala Asp Ala His His Ala Arg Ser Ser
195 200 205
Gly Tyr Gly Pro Val Leu Ser Leu Leu Thr Cys Phe His Phe Gly Arg
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<210> 43
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<213> Sulfolobus shibatae

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aatttatcta tatacgagaa agttagaaag agagaataaa atgagtgacg aattaagttc 240
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Glu Ala Ser Phe His Leu Phe Thr Ala Gly Gly Lys Arg Leu Arg Pro
35 40 45
Leu Ile Leu Val Ser Ser Ser Asp Leu Ile Gly Gly Asp Arg Gln Arg
50 55 60
Ala Tyr Lys Ala Ala Ala Ala Val Glu Ile Leu His Asn Phe Thr Leu
65 70 75 80
Val His Asp Asp Ile Met Asp Arg Asp Tyr Leu Arg Arg Gly Leu Pro
85 90 95
Thr Val His Val Lys Trp Gly Glu Pro Met Ala Ile Leu Ala Gly Asp
100 105 110
Tyr Leu His Ala Lys Ala Phe Glu Ala Leu Asn Glu Ala Leu Lys Gly
115 120 125
Leu Asp Gly Asn Thr Phe Tyr Lys Ala Phe Ser Val Phe Ile Asn Ser
130 135 140
Ile Glu Ile Ile Ser Glu Gly Gln Ala Met Asp Met Ser Phe Glu Asn
145 150 155 160
Arg Val Asp Val Thr Glu Glu Glu Tyr Met Gln Met Ile Lys Gly Lys
165 170 175
Thr Ala Met Leu Phe Ser Cys Ser Ala Ala Leu Gly Gly Ile Ile Asn

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            180                 185                 190
Lys Ala Ser Asp Asp Ile Ile Lys Asn Leu Val Glu Tyr Gly Leu Asn
        195                 200                 205
Leu Gly Ile Ser Phe Gln Ile Val Asp Asp Ile Leu Gly Ile Ile Gly
    210                 215                 220
Asp Gln Lys Glu Leu Gly Lys Pro Val Tyr Ser Asp Ile Arg Glu Gly
225                 230                 235                 240
Lys Lys Thr Ile Leu Val Ile Lys Thr Leu Ser Glu Ala Thr Asp Asp
                245                 250                 255
Glu Lys Lys Ile Leu Val Ser Thr Leu Gly Asn Arg Glu Ala Lys Lys
            260                 265                 270
Asp Asp Leu Glu Arg Ala Ser Glu Ile Ile Arg Lys Tyr Ser Leu Gln
        275                 280                 285
Tyr Ala Tyr Asn Leu Ala Lys Lys Tyr Ser Asp Leu Ala Leu Glu His
    290                 295                 300
Leu Arg Lys Ile Pro Val Tyr Asn Glu Thr Ala Glu Lys Ala Leu Lys
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<210> 47
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<400> 47
acgtggtgaa ctgccagtga 20

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Filed on 26 January 2001 (26.01.2001)
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Filed on 4 May 2001 (04.05.2001)

(71) Applicant *(for all designated States except US)*: **CARGILL, INCORPORATED** [US/US]; P.O. Box 5624, Minneapolis, MN 55440-5624 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **DE SOUZA, Mervyn, L.** [US/US]; 10935 38th Avenue North, Plymouth, MN 55441 (US). **KOLLMANN, Sherry, R.** [US/US]; 12031 99th Avenue North, Maple Grove, MN 55369 (US). **MAY, Colleen, A.** [US/US]; 20 Gideons Point Road, Tonka Bay, MN 55331 (US). **SCHROEDER, William, A.** [US/US]; 3509 Highlands Road, Brooklyn Park, MN 55443 (US).

(74) Agent: **DEGRANDIS, Paula**; Cargill, Incorporated, P.O. Box 5624, Minneapolis, MN 55440-5624 (US).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

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(54) Title: CAROTENOID BIOSYNTHESIS

(57) Abstract: Membranous bacteria that produce astaxanthin and other carotenoids are described, as well as isolated nucleic acids and expression vectors that can be used for producing carotenoids in microorganisms.

WO 02/079395 A3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US02/02124

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) : C07H 21/04; C12N 15/00, 15/63, 1/20; C12P 23/00
US CL : 435/67, 252.3, 252.33, 320.1; 536/23.1, 23.2, 23.7
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/67, 252.3, 252.33, 320.1; 536/23.1, 23.2, 23.7

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ARMSTRONG, G. A. Genetics of eubacterial cartenoid biosynthesis: a colorful tale. Annu. Rev. Microbiol. 1997, Vol. 51, pages 629-59, the entire document.	1-7, 39, 47, and 73-81
X	LIU, S.-T. Carotenoid-biosynthesis gene as a genes as a genetic marker for the purpose of gene cloning. Biochem. Biophys. Res. Commu. 31 August 1993, Vol. 195, No. 1, pages 259-263, see Figure 1 and abstract.	39
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A		1-7
X	US 5,965,795 (HIRSCHBERG et al.) 12 October 1999, see abstract.	39
---		---
A		47, and 73-77
A	US 5,429,939 (MISAWA et al.) 4 July 1995, see abstract.	1-7
---		---
X		39
A	US 5,811,273 (MISAWA et al.) 22 September 1998, see the entire document.	1-7, 47 and 73-81
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X		39

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
24 January 2003 (24.01.2003)

Date of mailing of the international search report
27 MAY 2003

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231
Facsimile No. (703)305-3230

Authorized officer
Nashaat T. Nashed
Telephone No. 703-308-0196

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

C. (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A --- X	US 5,684,238 (AUSICH et al.) 4 November 1997, see entire document.	1-7 and 81 ---------- 39
A	HANNIBAL et al. Isolation and characterization of canthaxanthin biosynthesis gene from the photosynthetic bacterium Bradyrhizobium sp. strain ORS278. J. Bacteriol. July 2000, Vol. 182, No. 13, pages 3850-3853, see Figure 1.	1-7
A	MISAWA et al. Structure and function analysis of a marine bacterial carotenoid biosynthesis gene clustur and astaxanthin biosynthetic pathway proposed at the gene level. J. Bacteriol. November 1995, Vol. 177, No. 22, pages 6575-6584.	1-7, 39, 47, and 73-81
A	MISAWA et al. Elucidation of the Erwinia uredovora carotenoid biosynthetic pathway by functional analysis of gene products expressed in Escherichia coli. J. Bacteriol. December 1990, Vol. 172, No. 12, pages 6704-6712, see the abstract.	1-7, 39, 47, and 73-81
A	TO et al. Analysis of the gene cluster encoding carotenoid biosynthesis in Erwina herbicola Eho13. Microbiology 1994, Vol. 140, pages 331-339, see entire document.	1-7, 39, 47, and 73-81

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US02/02124

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.: 1-7,39,47 and 73-81

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.
☒ No protest accompanied the payment of additional search fees.

Continuation of B. FIELDS SEARCHED Item 3:
Sequence search of SEQ ID NO's: 1, 2, 38 and 39 in commercial data bases, published U. S. applications files, and issued US patents. STN search (data bases): Medline, Caplus, Scisearch, lifesci, Biosis, and Embase. WEST (data bases): USPT, PGPB, JPAB, EPAB, and DWPI.